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EFFECT OF ACTIVATION ON THE WATER PERMEABILITY OF SALMON EGGS[1]

DAVID M. PRESCOTT
Department of Zoology, University of California, Berkeley, California

FIVE FIGURES

INTRODUCTION

With respect to their osmotic environment eggs fall naturally into two classes; those which are essentially in osmotic equilibrium with the surrounding medium such as eggs of marine invertebrates, and those which show a considerable degree of independence of the osmotic environment, represented by amphibian, avian, reptilian, and teleost eggs. Possibly in this latter class fall also the eggs of fresh water invertebrates but practically no information is available for this group.

For continued life and activity of all cells it is essential that the internal salt concentrations remain within some particular limits. In such a dilute environment as fresh water there are principally two possible mechanisms whereby developing eggs could maintain the necessary internal milieu; (1) by the performance of osmotic work involving active excretion of water and active uptake or passive retention of ions, (2) development of a closed system with a complete barrier to passage of water or ions across the surface. Although situation (1) has been amply demonstrated for certain adult cells, there is no physiological evidence that egg cells carry on such activities. On the other hand, a fair number of reported observations and experiments attest to the fact that the activated eggs of a number of different teleosts, both marine and fresh water forms, are completely impermeable to water and solutes. In a paper

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[1] Equipment obtained under a grant from the American Cancer Society was generously loaned by Dr. Daniel Mazia for this work.

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published in 1894, Jacques Loeb made note of the normal development of eggs of the marine and brackish water teleost, *Fundulus,* in distilled water or concentrated sea water without any apparent changes in egg volume. Using electrical conductivity methods Brown, in 1905, was unable to detect any loss of ions from *Fundulus* eggs developing in distilled water. The *Fundulus* egg has since been frequently used in embryological studies, and many investigators, including the author, have been impressed with its remarkable independence of external osmotic conditions. Other studies on activated eggs of different species of trout (Bogucki, '30; Gray, '20, '32; Krogh and Ussing, '37; Prescott and Zeuthen, '53; Svetlov, '29) and of the salmon (Hayes, '49; Hayes and Armstrong, '42; Manery and Irving, '35) lead to the conclusion that the activated eggs of these teleosts are water and ion impermeable, and in spite of the clear-cut experiments of Gray ('20, '32) on the brown trout egg demonstrating that the impermeability resides in the plasma membrane and not the chorion, some biologists have remained unconvinced. Recent studies (Kanoh, '52; Kusa, '51) on unactivated eggs of the salmon demonstrating permeability of the plasma membrane to water make it necessary to emphasize the difference between unactivated and activated eggs. The experiments of Krogh and Ussing ('37) using heavy water as an indicator of water permeability suggest that activation of trout eggs, in addition to visible changes, also involves a change of the plasma membrane from a water permeable to a water impermeable structure. The experiments described below, in which advantage has been taken of the highly sensitive heavy water-diver balance technique (Løvtrup and Pigón, '51; Pigón and Zeuthen, '51; Prescott and Zeuthen, '53), were designed to demonstrate such a change in the permeability state of salmon *(Oncorhynchus tshawytscha)* eggs during the process of activation.

METHODS

The cartesian diver balance (Løvtrup and Zeuthen, '51; Zeuthen, '48) was used to measure the heavy water (D_2O)

permeability of single salmon eggs in a manner described originally by Pigón and Zeuthen ('51) and as employed for permeability studies by Løvtrup and Pigón ('51) and Prescott and Zeuthen ('53). The method offers several advantages over osmotic methods; (1) it does not involve measuring volume changes, which are difficult except in the case of cells of regular geometric shape, (2) the plasma membrane is neither stretched nor shrunk nor does the method involve the formation of new membrane, (3) experiments can be carried out in an isotonic medium thus avoiding the complication of salt leakage.

No effects on the normal development of fertilized eggs immersed in 20% D_2O continuously could be detected.

The change in weight of single eggs immersed in a 15% D_2O medium was followed on the diver balance. Reduced weights of eggs and the standard were calculated from equations described in Zeuthen's paper ('48). In this investigation a small strip of platinum was used for a standard weight. The permeability constants were determined using equation (3) from Prescott and Zeuthen ('53). The water volumes of the eggs were calculated from the equation,

$$V_{H_2O} = \frac{\Delta RW\infty}{d_1 - d_2} \qquad (1)$$

in which ΔRW_∞ is the change in reduced weight of the egg after infinite time in the heavy water medium, d_1 is the density of the heavy water medium, and d_2 is the density of the same medium without heavy water. In those experiments in which water permeability was demonstrated, the heavy water exchange was more than 99% completed by the end of one hour, and the ΔRW after one hour in the heavy water medium can be substituted for ΔRW_∞ in equation (1). The water volume of a cell can also be determined by subtracting the dry weight from the wet weight (density × volume). The results by the two methods agree within 2% (Prescott and Zeuthen, '53). However, water volume by the heavy water indicator method does not involve sacrificing the cell in order to determine the dry weight.

The density of a cell can be calculated from the equation,

$$d_c = \frac{RW}{V_c} + d_m \qquad (2)$$

in which RW is the reduced weight of the cell in a medium of density d_m, and V_c and d_c are the volume and density of the cell.

The diver balance was constructed essentially as described by Zeuthen ('48). The equilibrium pressure for the empty diver balance changed at a constant rate of 4 mm of water pressure per 24 hours indicating an apparent increase in weight of the diver. The effect was due to a slow loss of air from the diver, probably by diffusion through the tail since after several weeks the air volume of the diver had decreased by a detectable amount.

MATERIALS

Pacific salmon eggs *(Oncorhynchus tshawytscha)* were obtained from the U. S. Fish Hatchery, Coleman Station, Anderson, California. Permeability studies were made on unfertilized, unactivated eggs and unfertilized and fertilized, activated eggs. Unactivated eggs were collected directly from the coelom of the female adult into a physiological salt solution (fish Ringer's) (0.09 M NaCl, 0.28 M KCl, 0.023 M $CaCl_2$, 0.010 M $MgCl_2$). Activated eggs, fertilized and unfertilized, were collected directly into river water (F.P. = – 0.02°C.). All eggs were stored at 15°C. and the experiments were carried out at this temperature.

EXPERIMENTAL RESULTS

Eggs collected from the adult coelom directly into fish Ringer's do not undergo activation (water hardening) until transferred to river water. Unactivated eggs are clear, deep orange, easily deformed by slight mechanical pressure, and possess a chorion which is soft and pliable. Such eggs were not fertilizable in Ringer's but a small percentage could be fertilized, after 6 hours storage in Ringer's, when transferred to river water containing a high concentration of sperm. The sperm were not freshly collected, and this, in part, may account for the low fertilization.

Unactivated eggs can be activated by transfer to a strongly hypotonic medium such as tap water (F.P. = – 0.01°C.). The process of activation is independent of whether or not fertilization occurs. After three to 4 minutes in tap water the egg is noticeably larger due to water intake into the newly formed perivitelline space. Swelling continues for about 40 minutes at which time eggs have increased about 15% in total volume. The chorion has changed to a tough and inelastic structure, and the egg itself is now strongly resistant to deformation.

In contrast with unactivated eggs the surfae of activated eggs is diffusely white and opaque. Breaking the egg open in Ringer's reveals that the contents are still clear orange in color. The opacity of the surface is probably related to the effect noted by Gray ('20) in which the proteins of the yolk of brown trout eggs are precipitated by weak salt solutions (such as tap water), the yolk turning from a clear to a white, opaque mass.

Heavy water permeability. An unactivated egg was placed on a diver balance in Ringer's containing 15% D_2O and the weight changes due to D_2O exchange with H_2O of the egg were recorded. The weight changes are presented graphically in figure 1 (curve I) which shows that D_2O exchange was essentially complete by the end of 60 minutes. There was always about a one minute delay from the time at which the egg reached the D_2O medium until the manometer could be adjusted and the diver brought into position for the first equilibrium pressure reading. The change in reduced weight of the egg occurring during the first minute was found by plotting the logarithm of the changes in reduced weight during succeeding intervals against time (fig. 2) and extrapolating back to zero time. Ten such experiments were performed, and the permeability constants calculated from equation (3) in Prescott and Zeuthen ('53) are given in table 1.

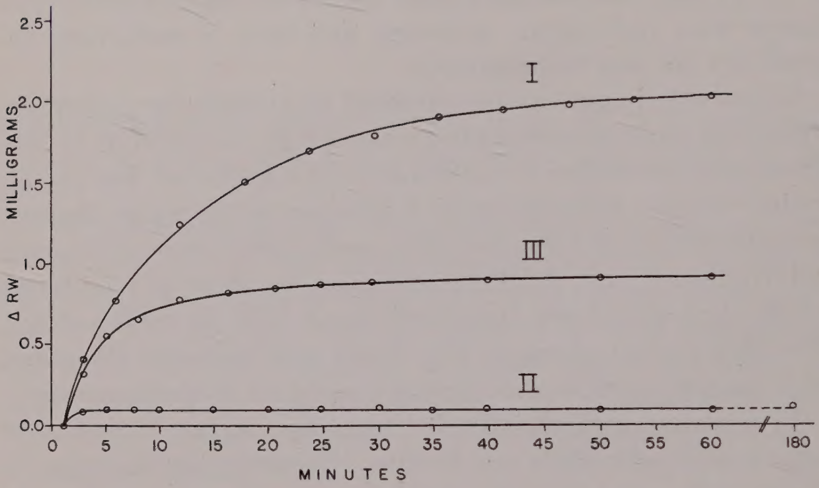

Figure 1 Curve I. Increase in the reduced weight of a single unactivated salmon egg in Ringer's containing 15% heavy water. Curve II. Change in the reduced weight of a single unfertilized, activated salmon egg in river water containing 15% heavy water. Curve III. Increase in the reduced weight of a salmon egg undergoing activation in river water containing 15% heavy water.

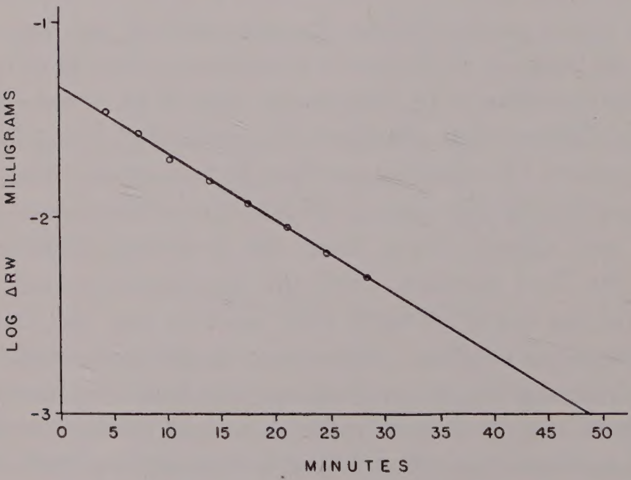

Fig. 2 Logarithm of the change in the reduced weight of an unactivated salmon egg in Ringer's containing 15% heavy water.

Just as with unactivated eggs 10 experiments were carried out with activated eggs, five with eggs in early cleavage stages and five with unfertilized eggs. In all cases the result was the same; activated eggs placed on a diver balance in river water containing 15% D_2O showed a slight increase in weight during the second minute [2] and no further increase during the following three hours at the end of which time six of the experiments were terminated. The results of a typical experiment are plotted in figure 1 (curve II) for direct comparison with the weight changes of an unactivated egg in 15% D_2O (fig. 1, curve I).

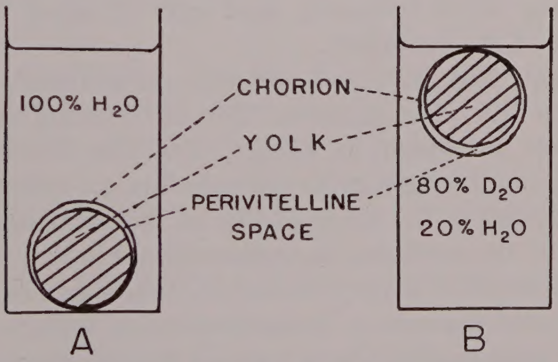

Fig. 3 Demonstration of chorion permeability to heavy water. A. Activated salmon egg in plain water with the perivitelline space at the top of the egg. B. Activated salmon egg floating with the yolk sphere floating within the chorion.

It seems likely that since there is a small weight increase during the second minute, a somewhat larger weight increase occurs during the first minute. This initial small but rapid weight change is attributed to exchange occurring between the water of the perivitelline space and the D_2O of the heavy water medium. To test the water permeability of the chorion, activated eggs (density = 1.056) were placed in 80% D_2O (density = 1.080). The eggs float in this medium but the bulk of the perivitelline space, which is normally at the top of the egg (fig. 3 A), now appears at the bottom (fig. 3 B). This means that the yolk mass, which is less dense than the heavy water medium, is supported by this medium while the chorion, which must have a density greater than 1.080, is not supported. The shift in the position of the perivitelline space occurs immediately on transfer from plain water to the heavy water medium or vice versa. To account for these observations the chorion must be freely permeabale to D_2O, and the heavy water which enters the perivitelline space causes the yolk mass to float within the chorion. For the yolk to remain floating it is necessary that the yolk membrane be impermeable to heavy water. Activated eggs were left in the heavy water medium for 24 hours during which time only dead eggs (2 out of 30) sank to the bottom of the container.

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[2] It was not possible to obtain weight recording during the first minute. See Methods.

Four eggs, two fertilized and two unfertilized, were each kept on the diver for 24 hours. The unfertilized eggs continued to show no change in weight. The two developing eggs evidence a small weight gain representing an exchange in one case of 5% and in the other of 6% of the total water volume with D_2O of the medium. It is uncertain whether this weight change is a result of a very limited D_2O exchange or a result of developmental changes or a combination of both.

Since it appears that in the process of activation the plasma membrane changes from a slightly water permeable to a completely water impermeable structure, experiments were made to learn how rapidly, during activation, the condition of impermeability sets in. Single unactivated eggs were transferred from Ringer's to a diver balance in river water containing 15% D_2O and weight changes followed. Since the swelling occurring with activation will involve uptake of both ordinary and heavy water in the same ratio in which they are present in the external medium, no increase in reduced weight should result from this phenomenon, but any exchange of ordinary water of the egg with D_2O of the medium will bring about a net increase in the reduced weight. During the initial 5 minutes the reduced weight increased but the rate of increase fell off rapidly reaching nearly zero by the end of 25 minutes. The experimental values are plotted in figure 1 (curve III). From the total change in reduced weight it was calculated that approximately 50% of the possible D_2O exchange had taken place before the egg had become a closed system. Thus the change to the impermeabale state, although not immediate, progresses at a rapid rate and is completed within 25 minutes.

TABLE 1

Permeability constants for water of unactivated and activated eggs

	EXP.	P_d	EXP.	P_d
		μ/sec.		μ/sec.
Unact.	1	1.60	6	1.92
	2	1.78	7	1.34
	3	1.61	8	1.77
	4	1.57	9	1.59
	5	1.64	10	1.84
		Average 1.68		
Activated, unfertilized		Average 0.00		
Activated, fertilized		Average 0.00		

TABLE 2

Various physical values for activated and unactivated eggs of the Pacific salmon, Oncorhynchus tshawytscha. Each value represents the average of measurements on ten eggs

	WET WT.	DRY WT.	VOLUME	H_2O PER CENT	DENSITY
	mg	*mg*	*mm*^{3}	%	
Unact. eggs	245	93	230	66	1.065
Act. eggs	283	93	268	71	1.056

Returning activated eggs to Ringer's had no visible effect and did not re-establish water permeability.

Activated eggs killed by heating to 33°C. for one minute were very permeable to heavy water. Gray ('20) has demonstrated that dying or dead eggs of the brown trout in tap water rapidly lose salts and as a result the yolk globulins precipitate, turning the egg to a white, opaque mass. The same appears to be true of salmon eggs.

DISCUSSION AND CONCLUSIONS

From the results of these and other experiments referred to throughout this paper dealing with permeability of various teleost eggs, the following description of conditions is presented. Teleost eggs spawned into fresh water are adapted for development in a strongly hypotonic environment by the possession of an impermeable plasma membrane. Before spawning the eggs are stored within the body cavity of the adult where they are in osmotic equilibrium with the coelomic fluid. Shedding of the eggs into fresh water, regardless of whether or not fertilization occurs, results in rapid changes at the egg surface generally referred to as activation or water hardening. The chorion, which is at first soft and elastic, becomes rigid and stiff at the same time increasing in area and lifting away from the yolk, thus forming the perivitelline space. The perivitelline space becomes filled with water from the surrounding medium as it is formed. Svetlov ('29) has suggested that the water uptake of the pervitelline space results from imbibition by protein released from the yolk surface. The chorion appears to be impermeable to colloids (Bogucki, '30; Svetlov, '29), and the protein of the perivitelline space must also be prevented from leaking out.

The proteins of the yolk are insoluble in very dilute salt solutions as can be simply demonstrated by crushing an egg in tap water. The whole yolk changes in appearance from clear orange to opaque white. In Ringer's the proteins do not precipitate. It is therefore probable that the change of the yolk surface from clear orange to diffuse white occurring when an egg passes from the isotonic environment of the coelom to fresh water is simply a precipitation of proteins at the yolk surface. Concomitant with the visible changes is the rapid decline and eventually complete disappearance of water permeability. The two events may be directly related; however, it seems unlikely that a layer of precipitated protein alone could present an absolute barrier to water, and either the precipitated material is in fact lipoprotein or there are other rearrangements at the yolk surface at activation involving part of the plentiful store of yolk lipid.

SUMMARY

The cartesian diver balance-heavy water method was used to measure water permeability of unactivated and activated (water hardened) salmon eggs and eggs in the process of activation. Unactivated eggs are slightly water permeable but become completely impermeable within 25 minutes after activation has begun. The chorion is freely permeable to water, and the impermeability of activated eggs resides in the plasma (yolk) membrane. The change to the impermeable state may be due to the precipitation of lipoprotein at the yolk surface.

ACKNOWLEDGMENTS

The author wishes to express his thanks to Mr. John Pelnar of the Coleman Fish Hatchery, Anderson, California for generously providing the salmon eggs, and to Dr. Erik Zeuthen for suggestions and criticisms concerning the experiments and the manuscript.

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THE MOLECULAR WEIGHT OF CYPRIDINA LUCIFERASE[1]

AURIN M. CHASE
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ONE FIGURE

The blue luminescent reaction of the marine ostracod crustacean, *Cypridina hilgendorfii,* occurs when the organism ejects into the sea water granules containing an enzyme, luciferase, and a compound called luciferin. Only luciferase, luciferin, oxygen and water appear to be necessary (Harvey, '52). The luciferase and luciferin can be extracted from dried, powdered organisms, allowing the luminescent reaction to be studied *in vitro.* The luciferin has been considerably purified by a method developed by Anderson ('35), while McElroy and Chase ('51) describe a means of partially purifying the enzyme.

Cypridina luciferase being a protein, it would be highly desirable to know its molecular weight. To this end, measurements have been made with the Spinco analytical ultracentrifuge[2] of the sedimentation constant of *Cypridina* luciferase solutions, prepared by the method of McElroy and Chase ('51). It is the object of this paper to describe these measurements and to present the resulting values of the molecular weight of the enzyme.

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[1] This work was supported in part by funds of the Eugene Higgins Trust allocated to Princeton University.

[2] Done at the Marine Biological Laboratory, Woods Hole, Mass. My thanks are due to Mr. Robert Hyde, in charge of the centrifuge, who not only operated the apparatus but also contributed much helpful and practical advice.

EXPERIMENTAL PROCEDURE AND RESULTS

The material used corresponded to Fraction II from the purification procedure of McElroy and Chase ('51). Three ultracentrifuge runs were made. In one, called Experiment A, the cell contained the luciferase solution exactly as prepared, having a protein concentration of the order of 0.3%.

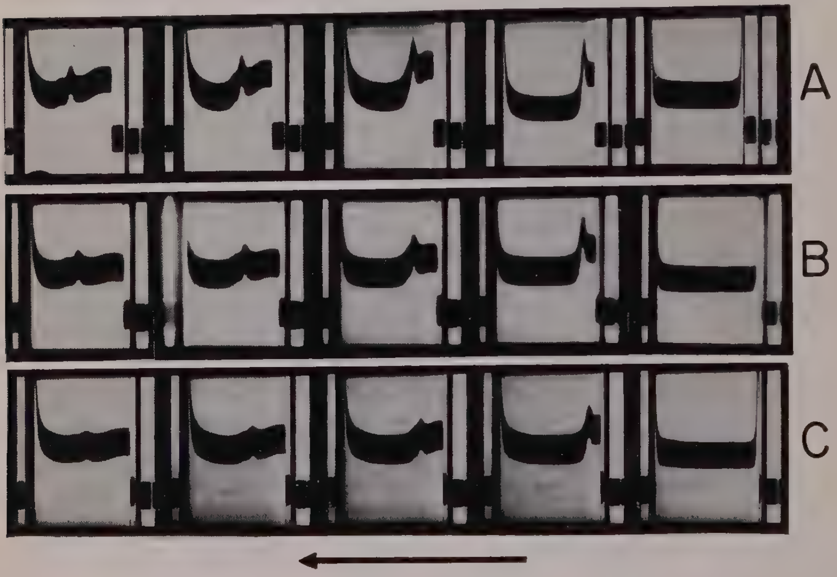

Fig. 1 Photographic records of three ultracentrifuge runs. In Experiment A the luciferase solution was used exactly as prepared. In B the concentration was reduced to one-half with water. The solution in Experiment C had been dialyzed against 0.02 *M*, pH 7.8 phosphate buffer containing sufficient sodium chloride to give an ionic strength of 0.1. Sequence of photographs and sedimentation are to the left, in the direction of the arrow.

Two components are evident in all three experiments. Luciferase activity is associated with the component of higher concentration. See text and table 1 for complete details.

For Experiment B the above solution had been diluted with an equal volume of distilled water. All components were consequently reduced to one-half their original concentrations. The solution used in Experiment C had been treated as follows: 5 ml of Fraction II were dialysed 24 hours against

2000 ml of 0.02 *M* phosphate buffer of pH 7.8, with sufficient sodium chloride added to make the ionic strength about 0.1.

In all three sedimentation runs photographs of the cell were taken at 32 minute intervals. Reproductions of these photographs appear in figure 1. Although the protein concentration is not optimal, there appear to be two components in each series of pictures, indicating that at least two high molecular weight compounds are present in the extraction. Although these components could not be isolated to determine which carried the enzyme activity, this information was obtained indirectly. Chase, Schryver and Stern ('48) showed that similar luciferase solutions, when studied electrophoretically at pH 7.5, possessed two moving components and a stationary one. In their experiments it was possible to identify the component of higher mobility as that carrying the luciferase activity. The solution used in Experiment B of the present work was also studied electrophoretically at pH 7.5 [3] and found to possess two moving components. The one with the higher mobility corresponded in concentration to that with the lesser sedimentation constant in the ultracentrifuge experiments. Identification was thus possible by indirect means.

CALCULATION OF MOLECULAR WEIGHT

Svedberg's standard relationship,[4] used in calculating molecular weights from sedimentation velocity data, also involves the value for the diffusion coefficient of the molecule under consideration. The diffusion coefficient of *Cypridina* luciferase is not known but, lacking this, a value of the molecular weight can still be calculated, assuming that the molecule be spherical and lack water of hydration. The viscosity of the protein solution must be known for this alternative method of calculation. The equation is as follows:

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[3] Done at the Marine Biological Laboratory with the portable Perkins-Elmer electrophoresis apparatus. I wish to express my thanks to Dr. Robert C. Warner for advice and help in the operation of this instrument.

[4] $M_{SD} = (RTs)/[D(1 - V\rho)]$, where M_{SD} is the molecular weight from sedimentation and diffusion data, s is the sedimentation constant, D the diffusion constant, V the partial specific volume (about 0.75 for many proteins), ρ density of the medium, R the gas constant and T is the absolute temperature.

$$M^2 = (162\pi^2 N^2 \eta^3 V s^3)/(1 - V\rho)^3,$$

where M is the molecular weight, η is the viscosity and ρ the density of the solution, N is Avogadro's number, V is the partial specific volume and s the sedimentation constant.

Although no proteins are known which are perfectly spherical molecules free of water of hydration, nevertheless, a calculation of the molecular weight of luciferase from the sedimentation constant alone seemed worthwhile. Viscosities relative to water were measured with an Ostwald viscosimeter at 26°C., since this was the prevailing temperature in the ultracentrifuge runs. Densities were also determined at this temperature. The partial specific volume was called 0.75.

TABLE 1

Calculated values of the molecular weight of luciferase, for the experimental temperature of 26°C., using the equation: $M^2 = (162\pi^2 N^2 \eta^3 V s^3)/(1 - V\rho)^3$.

The values of sedimentation constant, viscosity, density and pH are also shown in the table.

	EXPERIMENT A	EXPERIMENT B	EXPERIMENT C
Molecular weight	35,300	35,700	39,000
Sedimentation constant	2.89×10^{-13}	3.44×10^{-13}	3.56×10^{-13}
Viscosity	1.181×10^{-2}	1.024×10^{-2}	1.024×10^{-2} (assumed)
Density	1.0132 (calculated)	1.0051	1.0132 (assumed)
pH	6.5	6.5	7.5

Table 1 contains the calculated values of the molecular weight as well as the other factors used in the computations. The method of calculating the sedimentation constant is described in detail on pp. 382–387 of Greenberg ('51). In the present case the values have not been corrected for the difference between 26 and 20°C.

It can be seen from table 1 that the computed values of the molecular weight for the three solutions fall in the general range of about 35,000. This differs by a factor of less than 2 from the figure which was estimated by Kauzmann, Chase and Brigham ('49) as a value which gave reasonable results in calculating the thermodynamic constants for the formation and decomposition of the luciferase-luciferin complex. Johnson, Eyring and Polissar ('54) have compared thermodynamic constants, calculated from rate constants determined by Chase ('50) for the heat inactivation of *Cypridina* luciferase, with similar constants calculated by Kunitz ('48) for the heat inactivation of crystalline soybean trypsin inhibitor. They found striking similarity between the thermodynamic constants for these two systems, and since the molecular weight of the soybean inhibitor protein is 24,000, this is further indication that the molecular weight of luciferase may well be in this range.

An attempt was made to estimate the diffusion coefficient of the luciferase by measurements of the rate of spreading of the sedimenting boundary.[5] The protein concentration was, however, much too low to give reliable results by this method, as is apparent from the boundaries shown in figure 1. The value of 1.4×10^{-7} cm² per sec., obtained in this way for the diffusion coefficient, is probably of little significance. If used, it leads to a molecular weight 7 or 8 times as great as that already given and would indicate an unreasonably high degree of asymmetry for the molecule.

A reliable value of the diffusion coefficient of *Cypridina* luciferase is needed in order to check the value of the molecular weight from the present data. Diffusion measurements involving passage through sintered disks; etc., are probably not practical with luciferase because the enzyme suffers surface inactivation (Chase, unpublished observation) and its activity must be used as the criterion of concentration in

[5] The method is summarized in Greenberg ('51) on page 361.

diffusion measurements of this sort, since luciferase preparations are not yet free of other proteins. Probably measurements involving the spreading of the boundary would be the most practical, if sufficiently high concentrations of luciferase of adequate purity become available.

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SUMMARY

Solutions of *Cypridina* luciferase, purified by the method of McElroy and Chase ('51), have been studied in the Spinco ultracentrifuge. Lacking a knowledge of the diffusion coefficient of the enzyme, values of the molecular weight were calculated from the observed sedimentation constants (2.9 to 3.6. Svedberg units) measured under various conditions.

Assuming a spherical molecule lacking water of hydration, a minimum value for the molecular weight of luciferase of about 35,000 was obtained.

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AUTORADIOGRAPHS OF HAMSTER SPERMATOZOA [1]

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THREE FIGURES

Animal spermatozoa seldom have been referred to as suitable objects for autoradiographic studies. Bishop ('49, '51) has reported results with epididymal sperm cells of the bull, and Howard and Pelc ('50) discuss mouse spermatogenesis after radioactive phosphate treatments. This report presents some results of attempts to study hamster spermatozoa by P^{32} autoradiography. Numerous discussions of theories and methods of this technique have been published by Gross, Bogoroch, Nadler, and Leblond ('51), Gomberg ('51), Pelc ('51), and more recently by Doniach, Howard, and Pelc ('53).

Adult young male hamsters received intraperitoneal injections of $H_3P^{32}0_4$ that ranged between 60 and 120 μC in radioactivity. As a routine procedure, the standard dose was adjusted to approximately 1 μC per gram body weight. However, a range of experimental treatments were also tested, extending from 0.5 μC to as much as 3 μC per gram body weight. Autopsy was made after different time intervals up to twelve days following injection, and the testes and epididymides were removed for radioactivity determinations. Some fragments of testis tissue were used for routine microscopic study, and others were used for the preparation of autoradiographs.

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Autoradiographs were attempted by numerous methods, ranging from the use of sectioned testes prepared by standard and freeze-drying histological procedures to the use of isolated single cells prepared by teasing testis fragments in isotonic saline solutions with standard smear techniques. Washed and unwashed cells were used, as were distilled water suspensions. Boyd ('48) and Bishop ('49) both used the method of pipetting a solution of suspended cells directly onto the photographic emulsion of Eastman medium lantern slide plates. This method is the one used to obtain the autoradiographs referred to later. Dental x-ray film and NTB slides were also tried, but with less satisfactory results. Stripping film also has been used in later studies. Smears on glass slides were clamped tightly to the emulsion surface, but results were unsatisfactory due to difficulties arising in the recognition of any autoradiograph and its matching cell. Attempts to apply the Gomberg ('51) wet collodion method likewise did not prove successful in our experience.

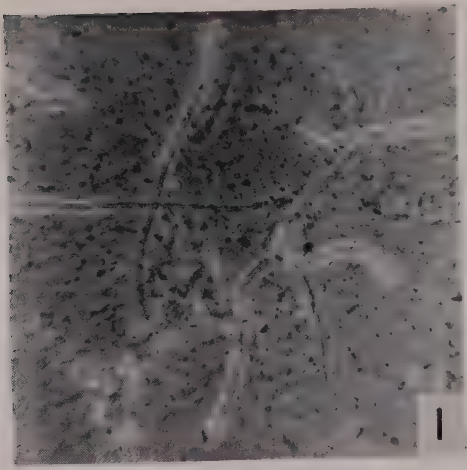
In sectioned materials, autoradiographic reactions with P^{32} have not been localized sufficiently sharply to be of cytological value. Granulation of the emulsion occurs, but it is diffuse and general over the section, as has been reported by Warren and Dixon ('49) who used the chicken testis with labelled phosphate (P^{32}).

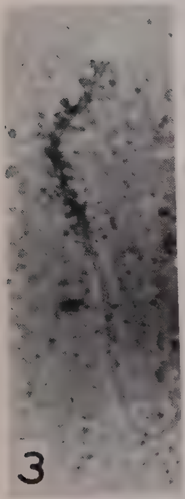
The general utility of a photographic emulsion as a rapid method for the preparation of isolated cells for microscopic examination is not fully recognized. Suspensions of germ cells and spermatozoa may be dried quickly without being subjected to chemical action other than that due to the media and the emulsion. After a few hours or overnight in the dark room, the photographic plate is developed by usual procedures and the cells may be studied with the phase microscope at later and more convenient times. Excellent definition of internal detail can be obtained without the necessity of staining and the emulsion preparation is permanent for long periods. A cover-slip may be added after a drop or two of a gelatin-chromalum mixture on the emulsion as recommended by Pelc ('51) in order to use oil immersion objectives. For 4 mm high dry examination, no cover-slip is needed. Various effects may be observed with the employment of different fluid media for the cell suspension (Crossman, '53). These methods seem worthy of exploration with a greater variety of materials and media.

Figures 1, 2 and 3 illustrate results obtained with epididymal spermatozoa of the hamster from the same autographic preparation and following similar treatment throughout. Tyrode's solution was employed as the suspension medium. It is observed that a well-defined reaction coincides with the large mid-piece regions of the spermatozoa in figures 2 and 3. This region is not uniform for all spermatozoa, as seen in figure 1, where no reaction can be seen. The latter also illustrates the usual negative result when distilled water, saline, or washed suspensions are used. Not figured are intermediate weak reactions between the two extremes. It is evident therefore, that the epididymal sperm population is not uniform. It seems of interest to consider the different degrees of the autoradiographic reaction observed, and their probable explanation.

The method of directly embedding cellular objects in a photographic emulsion for autoradiographic purposes is subject to question because of possible unknown or unpredictable chemical effects, such as reported by Board ('52) to occur with sulfhydryl compounds, and discussed further by Everett and Simmons ('53). Nevertheless, the method seems practicable for some purposes, and seems worthy of further study and use in suitable biological situations.

The results obtained with spermatozoa by this method might be closely allied to the von Kossa ('01) test for calcium in tissue studies. Since no autoradiographic reactions occur when distilled water and normal saline suspensions are used, but do appear to a slight degree with Ringer's and are even better with Tyrode's as a suspension medium, an obvious dif-





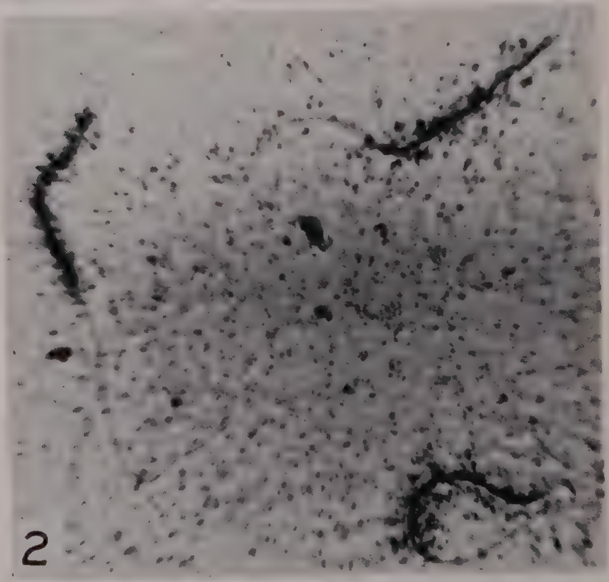


Fig. 1 Under the phase microscope with no apparent autoradiographic reaction. (× 290). Fig. 2 Under the ordinary microscope showing a strong autoradiograph in the mid-piece regions. Less uniformity is observed in those at the upper and lower right. (× 485). Fig. 3 The same spermatozoan as the one at the left of figure 2, but under the phase microscope. The finer and sharper delineation of the silver grains is apparent. (× 485).

Microphotographs of hamster epididymal spermatozoa from the same autoradiographic preparation after removal from an animal receiving $1.2\ \mu\text{C}\ \text{P}^{32}$ per gram body weight by a single injection 7 days prior to autopsy. Two hours elapsed before the preparations were made, and an exposure period of 56 days was permitted before development.

ference is to be found in the calcium content of Ringer's, and the calcium, magnesium, carbonate, and glucose content of Tyrode's. According to Lison ('36), the calcium test consists of the appearance of a blackening reaction that occurs when calcium phosphate is treated with silver nitrate and exposed to light. When calcium phosphate and silver nitrate are mixed directly a yellowish precipitate of silver phosphate is formed without a black coloration. This precipitate is rapidly reduced to give a black coloration, however, in the presence of albuminoid substances. Cameron ('30) has observed similar reactions occurring in purified gelatin media after the use of strontium, barium, copper, iron, magnesium, and carbonates, and emphasized that the reaction is not completely specific for calcium only. Suitable precautions should be taken to eliminate each possible factor that may be involved.

Since gelatin is an indispensable part of a photographic emulsion, it is conceivable that its components may serve to enhance the reaction, as in Cameron's model tests, and as albuminoids do. Careful study of figure 2 (specimen in lower right) reveals small clear vesicular areas along the mid-piece membranes suggesting diffusion associated with dehydration, and some seepage of organic substances could provide the suitable substrate for a surface precipitation reaction. It is not obvious by this method, however, that such a precipitation reaction has occurred, but neither can it be totally ruled out by examination of the autographs alone. The main arguments against the ocurrence of precipitation phenomena seem to rest in the fact that the reaction is not uniform for all spermatozoa, and that the reaction is not rapid as in the calcium test with silver nitrate and albuminoids. A long exposure of 56 to 60 days is required to obtain the result under consideration. If, however, some precipitation has occurred, a reasonable explanation of the reaction would be found in an interaction of calcium and magnesium phosphate with the silver compounds of the emulsion and a subsequent reduction to metallic silver grains by the action of β-particle radiant energy emitted by the radio-active P^{32} present. The dimensions, form, and uniformity of distribution of the silver grains in figure 3 under the phase microscope appear to argue against precipitation reactions.

Since carbonates may be also a significant factor in the reaction referred to above, it seems possible that the production of carbon dioxide by metabolizing spermatozoa may be a factor for consideration. The use of oxidizable reserves by active sperm cells may readily result in the accumulation of surface carbonates to interact with the mineral ions and facilitate the reaction. In this way, the variable intensity of the reaction in different spermatozoa may be explained. It is of interest to note in this regard that the oxidation of lipid reserves seems to be typical of spermatoza from this region of the epididymis (Lardy and Phillips, '45). Again, however, the assumption of precipitation effects would be necessary.

It appears more probable, however, that adherent lipoidal substances from the cauda epididymi provide a base for phosphate surface reactions and the subsequent events. Testicular sperm have not given results of similar intensity, and washed preparations have been negative. The failure of some spermatozoa to give a positive autographic reaction seems to be best explained as due to the absence of required materials for continued metabolism, and epididymal secretions doubtless supply these. The density of the sperm suspension may be an important factor in determining the availability of these substances to the entire sperm population.

In spite of the variable factor discussed above that may be involved in the autoradiographs described, it seems evident that the presence of radioactive P^{32} as phosphate provides the energy source for the completion of the reaction. Absorbed phospholipid substances from the epididymis are shown to be localized on the mid-piece membranes of a certain percentage of the spermatozoa in sufficient quantity to energize a strong autographic reaction in the presence of calcium and magnesium ions. More elaborate precautions should be taken to eliminate some of the complex interactions that are obviously present. It might be of interest, for example, to perform the converse experiment utilizing radioactive calcium in lieu of phosphorus with similar results to be expected under corresponding and appropriate conditions, if calcium is involved. It seems evident that the presence of calcium and/or other minerals and organic compounds may serve to increase the probability of successful autoradiography when applied with critical controls in suitable biological situations.

Friedlander and Fraser ('52) have shown that calcium is located in the mid-piece membranes of the spermatozoa of the bull. Since lipoproteins and calcium are well-known constituents of cellular membranes, it is not improbable that phosphate localization observed by this method may prove useful in the study of biological surface reactions under selected conditions. The actual phosphate localization is of obvious value in relation to energy sources in sperm metabolism, and to the question of membrane surface action in motile processes. Blackshaw ('53) observes that calcium reduces motility of ram spermatozoa, but has no significant effect with bull or rabbit sperm.

It was observed in the microscopic study of the autoradiographs that the spermatozoa could readily be divided into three categories, namely: those giving no reaction (fig. 1), those giving mild or weak reactions, and those with pronounced activity (figs. 2 and 3). The following percentages were determined for each category, in the same order listed: 50%, 20%, and 30%. This differential seemed worthy of further study to determine if any correlation could be established wih motility.

Distinct behavioral differences occur among epididymal spermatozoa examined from several regions of this organ. Similarly distinct differences in the P^{32} uptake of these areas have been observed and will be described in another report now in preparation. A reasonably close correlation was observed between the percentages of motile spermatozoa and those giving the autoradiographs (table 1).

The table gives a summary of average motility percentages for a group of six normal males. Ringer-glucose solutions were used and the percentages were estimated at hourly intervals for a five-hour period, during which the sperm suspensions were maintained at body temperatures. Three degrees of motility are defined readily as (1) nonmotile, or inactive; (2) vibratile, or weakly active; and (3) active or progressive. The percentages in the column referring to the autoradiographic results most closely fit the observed percentages obtained two hours following removal. Usually the time required to complete the autographic preparations occupied more than one hour, because of other simultaneous operations, such as dissection, preparation for counting, microscopic examination, etc. Also mild reactions have been observed in the mid-piece regions of testicular spermatozoa, which are vibratile only. Supporting evidence may be cited, since distilled water suspensions produce immediate immobility and give no autographic result. It therefore may be concluded that the autoradiograph provides a visible index of phosphate activities associated with metabolic and motile functions, and further that these activities are localized in the mid-piece regions of the spermatozoa.

TABLE 1

Motility percentages of hamster epididymal spermatozoa

DEGREE OF MOTILITY	TIME AFTER AUTOPSY						AUTORADIOGRAPHS AFTER 1–2 HOURS
	Immediate	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	
	%	%	%	%	%	%	%
Active	56	37	27	19	11	. .	30; strong
Vibratile	14	19	23	27	23	6	20; weak
Inactive	30	44	50	54	66	94	50; none

Since the mid-piece consists principally of mitochondrial concentrates, it is not unexpected that phosphate activity is demonstrable. Only recently has it been recognized that the mitochondria appear to be the principal agents of the cell in relation to oxidative processes. Cameron ('51) states that "they are probably not completely autonomous with respect to respiration, since they must be supplemented with cofactors, such as adenosine triphosphate and Mg ion. In the cell in all probability a rapid interchange of these factors, substrates, and inorganic phosphate takes place between the cytoplasm and the mitochondria." The autographic preparations do not readily distinguish adsorbed surface phosphate from phosphate that could be incorporated in phospho-lipids or the ribonucleic acid of the mitochondria. It seems evident, however, that surface activities are more significant in this result, since washing removes the radioactive phosphate and no autoradiograph is obtained.

Recent observations by Di Stefano and Mazia ('52) have indicated P^{32} uptake in the ribonucleic acid constituents of the mid-pieces of sea urchin spermatozoa. While it is possible that the autoradiographs might be interpreted in this way, it is more probable that the autoradiographic reaction has been enhanced by the intervention of surface phosphate containing epididymal secretions still available to the active spermatozoa. That these epididymal secretions are important in the attainment of active motility seems well-established, since Lardy and Phillips ('45) suggest that the isocitric acid cycle of fat and carbohydrate metabolism provides energy sources for sperm motility. Bishop ('52) points out that mammalian sperm metabolize phospholipids and other oxidizable compounds, and also show aerobic and anaerobic glycolysis. His experiments with P^{32} uptake in short time intervals are interpreted as related to acid-soluble phosphorus fractions and to glycolytic and ATP activities. It is of cytologic interest to be able to demonstrate that sites of phosphate concentrations are associated with the mid-pieces of epididymal spermatozoa and the degree of motility. For the present, numerous questions relating to mechanisms of phosphate absorption and metabolism remain subject to further investigation and analysis (McElroy and Glass, '52).

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A PHYSICAL THEORY OF THE GROWTH OF AXONS

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FOUR FIGURES

The quantitative work of the last twenty years on the structure of normal and regenerating peripheral nerves has shown that certain regular relationships exist among the linear dimensions of nerve fibres. The mechanical basis of these relationships is unknown, and would be very difficult to investigate directly. The method here employed is to form mechanical hypotheses which can be expressed quantitatively by applying the results of classical physics. Incorrect hypotheses can then be eliminated by comparison with the quantitative anatomical data. In this way, successful mechanical treatments have been found for the following aspects of the structure of medullated axons: the thickness of myelin as a function of fibre diameter, the constriction of the axon at a node of Ranvier, the distribution of fibre size in nerve trunks, and the relation of internodal length to fibre diameter.

I. Myelin thickness. The thickness of the myelin sheath of medullated nerve fibres has been found to increase systematically with fibre diameter by a number of authors including Schmitt and Bear ('37), Gasser and Grundfest ('39), Taylor ('42), Sanders ('48), Evans and Vizoso ('51) and Thomas ('54). The ratio of axon to fibre diameter is found to increase with fibre diameter, rapidly at first, and then more slowly. The material examined has included the peripheral nerves of earthworms, shrimps, trout, frogs, rabbits and cats, and this relationship between fibre size and myelin thickness is undoubtedly a fundamental property of medullated nerves.

It is uncertain exactly how the myelin is laid down. In mammals it appears on fibres which exceed 1–2 μ in diameter, and in peripheral nerves, is usually associated with the presence of Schwann cells. There is some evidence that it is deposited by the axons rather than by the Schwann cells, for Simpson and Young ('45) found that somatic nerve fibres became medullated after regenerating into an anterior mesenteric nerve trunk, which normally contained unmedullated fibres, whereas the fibres of the latter remained unmedullated when they were allowed to regenerate into the somatic nerve trunk.

Structurally, the myelin sheath consists of concentric lamellae in which lipoid and protein substances alternate (Schmitt, Bear and Palmer, '41). It is however able to flow axially under pressure, and during degeneration to form globules in which it retains its birefringence (Young, '45, page 48, and Causey and Palmer, '52).

A hypothesis to account for the observed relation between myelin thickness and fibre diameter can be formed by considering the possible methods of growth of the myelin sheath. It must be supposed that the myelin lamellae are laid down at one of the external surfaces of the sheath, and that when one lamella has been completed, a new one begins to form next to it (see Ben Geren and Raskind, '53). Throughout the process of myelination the axon diameter and the total fibre diameter increase. If then the lamellae were formed on the outer (Schwann cell) surface, the sheath could increase in thickness if each lamella were formed at a slightly larger diameter than the previous one. For the axon to increase in diameter, it would be necessary for the innermost lamellae to be destroyed and removed. Even if this were possible, however, there is no obvious reason why the myelin thickness should then depend so consistently on the fibre diameter. If on the other hand the myelin lamellae were formed on the inner axonic surface they would be stretched as the axon increased in diameter and as new lamellae were formed underneath. When through this stretching the breaking strain of the myelin was reached (at the outermost lamella), the myelin would fragment and the lamella would be replaced by the next one from beneath. With this mechanism of growth the outer fibre diameter would be limited by the axon diameter at which the lamellae were formed and by the breaking strain of the myelin. Furthermore this mechanism could continue to replace the myelin lamellae after the fibre had reached its mature diameter.

It is found by calculation that this hypothesis leads to an equation in agreement with the quantitative observations. Subsidiary to this testable part of the hypothesis, it may be supposed that the material needed to form the myelin flows down the axon from the cell-body as part of the "trophic influence" exerted by the cell-body on its axon (Young, '42), and also that the fragmenting myelin on the outer fibre surface is removed by the Schwann cells and the systemic circulation.

In order to calculate the relation of myelin thickness to fibre diameter according to this hypothesis, consider a lamella formed at the axonic surface of diameter d and forced out to the fibre surface of diameter D. The strain in the lamella would be $\frac{D-d}{d}$, and the stress k times this, where k is Young's modulus of the myelin. The stretched myelin lamellae would make the pressure in the axon greater than the pressure outside the fibre by an amount which we may denote by P_m. The relaxation of this containing pressure P_m as the lamella reached the outer surface of the fibre would produce a further tension in the outermost lamella equal to $\frac{1}{2}P_mD$, for by Laplace's equation, the internal pressure is equal to the tension divided by the radius of curvature, in this case $\frac{1}{2}D$. If the thickness of the lamella is denoted by t, then the tension of $\frac{1}{2}P_mD$ is equivalent to a stress of $\frac{1}{2}P_mD\,t.^{-1}$. This outermost lamella is stressed to the critical breaking stress of the myelin, by hypothesis. Let us denote this critical breaking stress by S . Then:

$$S_c = k\frac{D-d}{d} + \tfrac{1}{2}P_m.D.t.^{-1} \qquad (1)$$

It remains to evaluate the pressure P_m, which is the sum of the contributions to the internal pressure of all the lamellae of myelin. Consider a lamella of diameter x lying between the two surfaces, so that $d < x < D$. Then the strain in the lamella is $\frac{x - d}{d}$, the stress *k* times this as before, and the tension *t* times the stress. The contribution that this lamella makes to the total internal pressure is therefore $2k. \frac{x - d.}{xd} t.$, by Laplace's equation. Then:

$$P_m = 2\,k \sum_{x = d}^{x = D} \frac{x - d.}{xd}\, t. \qquad (2)$$

Since the myelin lamellae are very thin compared to the total thickness of the myelin sheath the summation may be performed sufficiently accurately by integration. Let δx be the total thickness of a myelin lamella intercepted by each end of a diameter *x,* so that δx is equal to 2t. Then the approximation by integration is:

$$P_m = k. \int_d^D \frac{x - d}{xd}\, \delta x = k. \left(\frac{D - d}{d} - \log_e \frac{D}{d} \right) \qquad (3)$$

Substituting this value of P_m into equation (1) we obtain:

$$\frac{S_c}{k} = \frac{D - d}{d} + \tfrac{1}{2} D\, t^{-1} \left(\frac{D - d}{d} - \log_e \frac{D}{d} \right) \qquad (4)$$

Since the thickness of a myelin lamella is very small compared with the total thickness of the myelin sheath, $D\ t^{-1}$ is a large number, and the second term in equation (4) greatly predominates. We may accordingly neglect the first term, and obtain

$$\frac{2\,S_c\,t}{k} = D\left(\frac{D - d}{d} - \log_e \frac{D}{d} \right) \qquad (5)$$

$$= F\ (D, d),\ \text{say.}$$

Since the left hand side of equation (5) involves only structural constants of the myelin, and these are presumably independent of the diameter of the fibre in any one animal and nerve, the hypothesis may be expressed by saying that the right hand side of equation (5) should be constant for all values of *D* and *d* found in a given nerve. In table 1 the values of the right hand side of equation (5) are shown for some of the published data, in the column headed F (D,d).

TABLE 1

Measurements of axon and fibre diameters obtained by various authors together with the values of F(D,d) calculated from equation (5)

AUTHOR	D (μ)	d (μ)	F (D,d) (μ)
Thomas ('54)	4.0	2.0	1.23
Trout, lateral	6.0	3.3	1.32
line nerve,	10.3	6.0	1.82
largest	14.5	9.3	1.67
fibres in	17.2	11.3	1.76
fishes of	19.3	12.9	1.80
different	25.0	17.0	2.12
lengths.	28.6	19.8	2.20
	32.0	22.5	2.24
Evans and Vizoso			
('51)	2.75	1.50	0.625
Rabbit,	3.44	1.97	0.650
6 weeks old	4.18	2.14	1.19
N.G.M.	5.38	3.13	0.954
	6.13	3.50	1.17
	7.08	4.08	1.30
	8.32	5.29	0.998
	9.36	6.21	0.909
	10.13	6.69	1.02
Taylor ('42)	3.0	1.35	1.27
Cat,	4.0	2.28	0.769
saphenous and	6.0	4.2	0.430
sciatic	8.0	6.0	0.367
nerves.	14.0	11.2	0.376
	18.0	14.7	0.398
Thomas ('54)		Trout 8.0 cm long	
Trout,	3.09	1.42	1.22
lateral	7.86	4.32	1.74
line	13.08	8.50	1.41
nerve,		Trout 20.7 cm long	
sub-	3.09	1.39	1.74
maximal	7.56	4.25	1.53
fibres in	13.20	8.31	1.65
fishes of	18.6	13.0	1.35
three		Trout 54.5 cm long	
different	3.0	1.5	0.921
lengths.	7.56	4.28	1.49
	12.76	8.13	1.51
	19.63	13.44	1.60
	32.38	23.25	1.99
Sanders ('48)			
Rabbit,	2.0	0.64	1.97
peroneal	4.0	1.9	1.44
nerve.	6.0	3.4	1.18
	8.0	5.1	0.945
	14.0	10.0	0.889
	18.0	13.5	0.824

The data available to test equation (5) are not entirely self-consistent. In the measurements of Sanders ('48) and Taylor ('42) (taken from their figures 3 and 4) the ratio $\frac{d}{D}$ decreases more rapidly as D decreases than in the other data of Thomas ('54) and Evans and Vizoso ('51) (the latter were not published in this form in the original paper, but were kindly supplied by the authors). It will be seen from table 1 that the function F (D,d) calculated from the right hand side of equation (5) shows little systematic change of value for the data of Evans and Vizoso, but increases with D in the data of Thomas obtained from fishes of different lengths, and decreases with D in the data of Sanders and of Taylor. The function F (D,d) is very sensitive to small changes in D and d, and better apparent agreement with the observations could have been obtained by taking an average value of F (D,d) and then calculating D for given values of $\frac{d}{D}$ by equation (5). With the first of Taylor's measurements, $D = 3.0$, $d = 1.35$, $F(D,d) = 1.27$, an error of only $0.1\,\mu$ in the measurements of the apparent positions of each of the myelin boundaries would add or subtract $0.2\,\mu$ from the estimate of D, and subtract or add $0.2\,\mu$ to the estimate of d. Then with $D = 2.8$, $d = 1.55$, $F(D,d) = 0.60$, in other words only half its former value. As errors larger than this can easily arise through slight changes in the histological preparation or photography as discussed by Evans and Vizoso ('51), little significance can be attached to the magnitude of the change in the estimates of F (D,d). Since furthermore the drift in F (D,d) is in opposite directions in the two kinds of data, and is negligible in the data of Evans and Vizoso, it may be concluded that equation (5) and the hypothesis from which it was derived are on the whole consistent with the dimensions observed.

II. The constriction of the axon at a node of Ranvier. Medullated axons are constricted at each node of Ranvier by an in-turning of the myelin sheaths of the two adjacent segments. This constriction is of significance both for the saltatory theory of nerve impulse conduction (Rushton, '51) and for the theory that the axon is constantly supplied with axoplasm from its cell-body (Young, '45; Weiss and Hiscoe, '48). Measurements of nodal constrictions have recently become available (Hess and Young, '52), and can be used to investigate the mechanical basis of the constriction.

In the early stages of medullation there are wide gaps between the myelin sheaths at the nodes (Vizoso and Young, '48). The nodal constrictions appear later, as these gaps close, and this suggests that adjacent myelin sheaths are pressed together at the nodes. It is probable that adjacent segments are separated by an inturned pleat in the neurilemma at the node (Hess and Young, '52). The myelin is formed in concentric lamellae (Schmitt, Bear and Palmer, '41), and is able to flow along the axon under pressure (Causey and Palmer, '52). The myelin appears to wet the axon but not the Schwann tube, and during degeneration the myelin sheath breaks up into droplets under the action of surface tension (Young, '45).

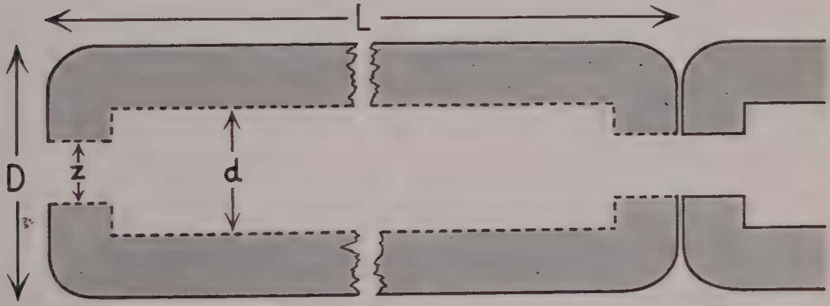

Fig. 1 A schematic representation of an axon between two nodes of Ranvier.

A hypothesis which accounts for the constriction of the axon at the node follows naturally from these facts, for surface tension forces should cause the myelin to flow into a hollow figure of minimal surface area. In order to calculate the consequences of this hypothesis it is convenient to apply the "principle of virtual work" and to consider a small radial displacement changing the diameter of the constriction at the node. During such a small displacement, the internodal length, the external diameter *D*, the volume of myelin and the volume of axoplasm in the segment must remain constant, and the internodal axon diameter must alter accordingly. Furthermore, a small displacement would not significantly change the surface area of the myelin, since this is a minimum. This situation is equivalent to disturbing some oil and water contained in a stoppered bottle to determine the shape of a stable boundary between the two liquids. Calculation shows that these consequences of the hypothesis imply a certain relationship among the dimensions of the axon at a node. This relationship is in agreement with the observed dimensions, provided that it is the area of the axonal surface where the myelin wets the axon which is minimised, and not the total or the external myelin area.

Text figure 1 is a simple representation of the geometry of a segment, in which z denotes the axon diameter at the nodal constriction and L the internodal length. The dotted lines represent the area A which is to be minimised according to the hypothesis. Then:

$$A = \pi d (L - D + d) + \frac{\pi}{2} (d^2 - z^2) + \pi z (D - d) \qquad (6)$$

and small displacements in d and in z must leave the area unchanged.

To express this condition, we may differentiate A with respect to one of the variables, say z, and put $\frac{dA}{dz} = 0$. Thus:

$$0 = \frac{dA}{dz} = (3d + L - D - z)\, \pi \left(\frac{dd}{dz} \right) + \pi (D - z - d) \qquad (7)$$

Let V be the volume of axoplasm in a segment, then:

$$V = \frac{\pi}{4} d^2 (L - D + d) + \frac{\pi}{4} z^2 (D - d) \qquad (8)$$

This volume is to be constant during the displacements of d and z, so:

$$0 = \frac{dV}{dz} = \frac{\pi}{4} (3d^2 + 2dL - 2dD - z^2) \left(\frac{dd}{dz} \right) + \frac{\pi}{4} (2zD - 2zd) \quad (9)$$

Eliminating $(\frac{dd}{dz})$ between equations (7) and (9),

$$(2dL - 2Dd + 3d^2 - z^2)(D - z - d) = (2zD - 2zd)(L - D + 3d - z) \qquad (10)$$

Now L is very much greater than any of the other dimensions of the segment, and the latter may be neglected where they occur with L.

Equation (10) then becomes:

$$\frac{d}{D} + \frac{z}{d} = 1$$

or (11)

$$z = \frac{D}{4} - \left(\frac{d - \frac{1}{2}D}{D}\right)^2$$

Equation (11) expresses the hypothesis so far developed, but a further possible relationship arises from the fact that the nodal constrictions would act as throttles, regulating the amount of axoplasm passing down the axon from the cell-body and entering or leaving any particular segment. Because of this it is possible that in the steady state the throttles would be fully opened, or fully closed. By inspection of the second form of equation (11) it will be seen that the expression on the right hand side has a maximum value when $d = \frac{1}{2}D$, so that $z = \frac{D}{4}$; there is however, no minimum value for z, since z decreases rapidly to zero as d increases. If the throttle is fully opened, then, there will be the subsidiary condition:

$$\frac{d}{D} = \frac{1}{2} = \frac{z}{d} \qquad (12)$$

Equations (11) and (12) can now be compared with the data. Table 2 shows the measurements of d, D, and z obtained by Hess and Young ('52) from the nodes of rabbit nerves in longitudinal section. According to equation (11) the sum of the ratios (z/d) and (d/D) should be equal to unity, and if the subsidiary throttle condition is satisfied [equation (12)], each of the ratios should be equal to one-half. From table 2, the mean value of the sum of the ratios is 1.032, and standard deviation of this mean is 0.0252. The predicted and observed values of the sum of the ratios differ therefore by only 1.25 times the standard deviation, and with 15 degrees of freedom this is not a significant difference. Equation (11) is therefore in accordance with the observations. Furthermore, the difference between the mean value of (z/d) and the predicted value of 0.5 is only 0.008, or 0.34 times the standard deviation of the mean. The mean of the observed values of (z/d) is therefore not significantly different from the value predicted by equation (12). However, the mean observed value of (d/D) is 0.539, and this differs from the predicted value of 0.5 by 3 times the standard deviation of the mean. The observed value of (d/D) therefore differs significantly from the value predicted by equation (12). Moreover, the individual values of (d/D) do not increase systematically with *D*, as do the corresponding values of the ratio measured between the nodes in cross-sections (see table 1), and the estimates of (d/D) are consistently lower than those found in cross-sectional measurements of rabbit nerves. It appears therefore that the ob-

TABLE 2

The nodal dimensions obtained by Hess and Young ('52) and the ratios of the dimensions which are to be compared with equations (11) and (12)

D (μ)	d (μ)	z (μ)	z/d	d/D	$\left(\frac{z}{d}+\frac{d}{D}\right)$
5.75	2.5	1.75	.700	.435	1.135
7.5	4.25	1.63	.384	.567	0.951
8.75	5.25	2.75	.524	.600	1.124
10.0	5.5	2.25	.409	.550	0.959
12.75	5.5	1.85	.336	.431	0.767
13.75	7.75	4.5	.581	.564	1.145
14.5	8.25	3.5	.424	.569	0.993
15.0	7.75	5.0	.645	.517	1.162
15.25	7.75	4.13	.533	.508	1.041
15.5	8.5	3.65	.429	.548	0.977
16.5	10.0	4.75	.475	.607	1.082
17.25	10.0	4.38	.438	.580	1.018
18.5	8.75	4.13	.472	.473	0.945
18.5	10.25	5.01	.489	.554	1.043
18.5	10.5	5.63	.536	.568	1.104
18.75	10.5	5.25	.500	.560	1.060
		Mean	.492	.539	1.032
	Standard deviation of mean		.0236	.0133	.0252

served value of (d/D) is a compromise between the value required by equation (12) for a maximum opening of the throttle at the node, and the value occurring between the nodes.

It may be concluded on the basis of the 16 measurements at present available that the dimensions of the axon at the node of Ranvier are determined by the tendency of the myelin sheath to form a surface of minimal internal area, with a maximal opening of the axon at the node.

III. The distribution of fibre size in nerve trunks. Nerve trunks differ in their fibre contents, some having both large and small medullated fibres distributed about two distinct modes, while others have medium and small fibres with a unimodal distribution. Fernand and Young ('51) have examined the distribution of fibre diameter in 25 different somatic nerve trunks. Mohuiddin ('51), Evans and Vizoso ('51) and Thomas ('54) have studied the development of bimodal distributions in the nerve trunks of growing animals. The cause of the differences in modality between nerve trunks is unknown, and there does not appear to be any simple relation between the modality and the function of a nerve trunk (Fernand and Young, '51). Nevertheless, fibre size is known to be correlated with the conduction velocity of the nerve impulse, and may also be related to the kind of functional connection made by the fibre.

Both normal and regenerating nerve fibres increase in diameter and mature only after they have made a suitable peripheral connection (Evans and Vizoso, '51; Sanders and Young, '45). The first fibres to reach the periphery become the largest, and fibres in motor nerves which are very late in reaching the periphery during regeneration are eliminated altogether, or at least lose their myelin, since the total number of medullated fibres in a nerve trunk decreases during the period of maturation (Sanders and Young, '45). A nerve trunk is surrounded by two membranes, the perineurium and the epineurium. The latter can be easily stripped off a nerve trunk (Causey and Palmer, '53), but if the perineurium is cut, the fibres rupture outwards (Sunderland, '46), and it appears that the perineurium exercises a considerable restraint upon the fibres it contains.

A hypothesis which gives a mechanical explanation of modality can be formed by supposing that the growth in diameter of nerve fibres is limited in part by the constraint of the perineurium. The fibres would then grow within the perineurium in the configuration giving the closest packing. The closest packing of equal cylinders is achieved when each one touches six neighbours (D'Arcy Thompson, '42, page 499), and this arrangement is illustrated in text figure 2 A. Any other arrangement such as that shown in figure 2 B is unstable unless the interstices between the cylinders are filled with smaller supporting cylinders. To develop the hypothesis for comparison with observation it will first be necessary to calculate the ratio of the diameter of the large cylinders to that of the small cylinders which would just fill the interstices in each of the configurations illustrated in figure 2. Let us denote the diameters of the large and the small cylinders by D_1 and D_2. Then by trigonometry, the ratio (D_1/D_2) is $(\frac{\sqrt{3}}{2-\sqrt{3}})$ or about 6.5 in the configuration of closest packing (A), and $(\frac{1}{\sqrt{2}-1})$ or about 2.4 in the other arrangement (B).

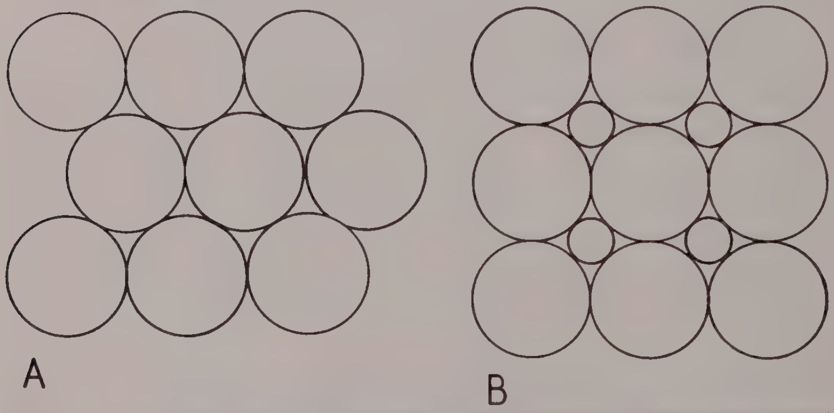

Fig. 2 The packing of equal cylinders. *A* the stable and closest arrangement, *B* an arrangement that is only stable if smaller supporting cylinders are present.

Nerve fibres become medullated at a diameter of 1–2 μ (Duncan, '34) and, in peripheral nerves grow inside Schwann tubes. The minimal diameter for a medullated nerve fibre in its Schwann tube is then of the order 2 μ. If fibres of 2 μ diameter filled the interstices of a nerve trunk arranged in the configuration of closest packing (A), the large fibres would have a diameter of 13 μ, since the ratio is 6.5 : 1. Nerve trunks having no fibres as large as 13 μ would not have sufficiently large interstices to accommodate medullated supporting fibres of 2 μ diameter, and would therefore have the stable configuration of closest packing (A) and a unimodal fibre distribution. If a nerve trunk has fibres of more than 13 μ diameter, however, the interstices could accommodate smaller supporting fibres. The configuration (B) would then be stable, and there would be a bimodal distribution of fibre size. The modal diameters of a bimodal distribution should be in the ratio 2.4 : 1, as calculated above.

A minimal diameter for a nerve fibre of less than 2 μ should be possible in central tracts such as the optic nerve where Schwann tubes are not seen. A bimodal distribution should then be possible in central tracts where the largest fibres are less than 13 μ in diameter, but the modal diameters should still bear the ratio 2.4 : 1.

The predictions of the hypothesis may be compared with the published observations set out in table 3. It is seen that the ratio of modal diameters is not significantly different from the value of 2.4 predicted, that the largest fibres of unimodal nerves seldom exceed 14 μ in diameter, and also that a growing nerve trunk first appears bimodal when its largest fibres are about 13 μ in diameter. These results are in surprisingly good agreement with the hypothesis since the nerve trunks contain fibres distributed on each side of the modes, and not fibres of one or two diameters only as considered in the calculation. According to the hypothesis, however, there should be equal numbers of fibres in each of the modes of a bimodal distribution, since there are as many interstices as cylinders. This is often not the case, and several small fibres may sometimes be seen occupying a space equivalent to one of the large fibres. A more serious objection to the hypothesis is that histological preparations (such as those reproduced in the publications cited above) of peripheral nerve trunks do not show the regular packing of fibres postulated.

TABLE 3

The distribution of fibre size in nerve trunks

BIMODAL NERVES			
	Upper modal diameter (µ)	Lower modal diameter (µ)	Modal ratio
Fernand and Young ('51)			
Muscle innervated			
tens. fasc. fem.	17	7	2.43
quad. fem. (2)	15	7	2.14
(1)	17	7	2.43
semimembranosus	17	7	2.43
semitendinosus	17	7	2.43
crureus	15	7	2.14
medial head of (1)	13	7	1.86
gastrocnemius (1)	17	7	2.43
lateral head of gastrocnem.	17	7	2.43
plantaris (1)	15	8	1.88
(1)	17	7	2.43
tibialis anticus	15	9	1.67
flex. longus digit. (2)	17	7	2.43
serratus anterior (1)	13	7	1.88
(1)	21	9	2.33
(1)	16	7	2.29
rhomboid (1)	19	7	2.71
(1)	17	7	2.43
long head of (1)	15	8	1.88
triceps brachii (1)	17	7	2.43
		Mean	2.26
		Standard deviation of mean	0.18
Bishop ('53)			
Optic nerve (cat)	6.5	3	2.17
UNIMODAL NERVES			
	% of fibres exceeding 14 µ diameter		
Fernand and Young ('51)			
Muscle innervated			
interosseous pedis (2)	0		
depress. labii inferioris	0		
sternothyroid	1.1		
phrenic	3.0		
recurrent laryngeal	0		
TRANSITION FROM UNIMODALITY TO BIMODALITY DURING GROWTH			
	Diameter of largest fibres at transition		
Mohuiddin ('51)			
(inferior dental nerve of cat)	about 13 µ		
Evans and Vizoso ('51) (rabbit N.G.M.)	about 12 µ		
Thomas ('54) (trout lateral line nerve)	about 13 µ		

In these preparations the outer fibres seldom appear to be in direct contact with each other or with the perineurium, although in vivo they rupture out of a cut in the perineurium (Sunderland, '46). It is possible that both these effects are due to differential shrinkage of the components of a nerve trunk during the process of histological preparation, and to loss of elasticity in the containing perineurium during fixation. It should be noted that this mechanical hypothesis by no means excludes a relation between the size of a fibre and its type of functional connection. The first fibres to reach the periphery come to occupy the upper mode, and may monopolise certain functional connections, or find only certain functional connections as yet developed. Later fibres will occupy the lower mode, and may have a smaller or a different choice of termination. The diameter that a fibre ultimately reaches may therefore be related to the attractiveness of its end connections to growing fibres, or to the time at which the end connections develop. There could then be a correlation between fibre size and function, even though the former were determined mechanically.

IV. The internodal lengths and diameters of nerve fibres. In the nerve trunk of a growing animal the diameter of the largest fibre increases systematically with the length of the nerve trunk (Thomas, '54). At the same time the internodal length of the largest fibre increases *proportionately* with the length of the nerve trunk (Ranvier, 1872; Thomas and Young, '49), implying that the number of nodes of Ranvier does not change after medullation. At any one length of the nerve trunk, the larger fibres have the longer internodal lengths (Ranvier, 1872, 1875; Thomas, '54). Over not too great a range of fibre diameters, the internodal length is roughly proportional to the fibre diameter (Thomas and Young, '49). The gradient of the line relating internodal length to fibre diameter increases with the length of the nerve trunk (Vizoso and Young, '48; Thomas and Young, '49). Over a wide range of diameters of fibres and lengths of nerve trunk, the curvature of the line relating internodal length to fibre diameter changes with the length of the nerve trunk (Thomas, '54). The factors determining the diameter of nerve fibres, and the rate of increase in diameter with length, remain uncertain; the subject has been reviewed by Thomas and Young ('49) and others.

In somatic nerve trunks, fibres become medullated at a diameter of 1–2 μ (Duncan, '34) with sheaths of a fixed length of the order 200 μ (Vignal, 1883; Thomas and Young, '49). Evidence has been presented that nerve fibres increase in length and diameter by an outpouring of axoplasm from the cell-body (Young, '45; Weiss and Hiscoe, '48). It is possible that the constraint of the various connective tissue sheaths is the main factor limiting the diameter of nerve fibres, and the role of the myelin (section I), the neurilemma (section II), and the perineurium (section III) has already been mentioned. The perineurium grows isometrically, its diameter increasing proportionately with its length (Thomas, '54).

It will now be shown that the hypothesis that the diameter of nerve fibres is limited by the constraint of the myelin, the neurilemma and the perineurium is consistent with the quantitative data available. It follows from the hypothesis that the turgor pressure in the axon is sufficient to outweigh the restraining pressure due to the myelin, and to stretch the neurilemma and the perineurium beyond their "natural" or unstretched diameters, so introducing two further restraining pressures. Furthermore, since these are the only restraints on the growth in diameter of the axon which are envisaged by the hypothesis, it follows that the turgor pressure in the axon is equal to the sum of the three restraining pressures on the axon. Some statement about the size of the turgor pressure in the axon must now be added to the hypothesis. It is simplest to suppose that the turgor pressure is determined biochemically in the cell-body, and is therefore independent of the dimensions of the axon, and is the same from one axon to another, at least within a homogeneous nerve trunk. Finally, a statement about the growth in diameter of the neurilemma must be added to the hypothesis, and it is reasonable to suppose that the neurilemma grows isometrically, like the perineurium (Thomas, '54), or in other words that the natural unstretched diameter of the neurilemma increases proportionately with the length of the nerve fibre it contains. A calculation based on this hypothesis leads to formulae which are in agreement with the published data.

The following notation will be used in the calculation in addition to symbols which have already been allotted in the three previous sections:

P_a = Turgor pressure in the axon.
P_n = Restraining pressure on the axon due to the neurilemma.
P_p = Restraining pressure on the axon due to the perineurium.
T = Natural, unstretched diameter of the neurilemma.
T_n = Natural, unstretched diameter of the neurilemma when the myelin is first formed underneath it.
Q = Natural, unstretched diameter of the perineurium.
Q_o = Natural, unstretched diameter of the perineurium at some length G_1 of the nerve trunk.
U = Effective, stretched diameter of the perineurium.
k′ = An elasticity constant of the neurilemma, = Young's modulus × thickness.
k″ = An elasticity constant of the perineurium, = Young's modulus × thickness.
L_o = The minimal internodal length at which medullation first occurs.
G = Length of nerve trunk.
G_{oD} = Length of nerve trunk at which a particular fibre of diameter *D* first became medullated.
N = Number of nodes of Ranvier.
R = A numerical constant related to the number of fibres in the upper mode.

The suffix *w* will be used to denote a quantity appropriate to the largest fibre in the trunk, and similarly the suffix *D* for a fibre of diameter D.

In terms of this notation, the hypothesis may now be expressed as:

$$P_a = P_n + P_p + P_m \qquad (13)$$

and the last term has already been evaluated in equation (3). The procedure will be to evaluate the other terms as functions of the stretched and unstretched diameters of the sheaths, and then use the part of the hypothesis postulating the isometric growth of the sheaths to express the unstretched diameters in terms of the observable fibre diameter, internodal length, and length of nerve trunk. An equation relating the observable quantities and the constants will thus be obtained.

To evaluate the pressure due to the neurilemma, we note that the effective stretched diameter of the neurilemma is equal to *D,* the outer diameter of the fibre. Then the strain is $\frac{D-T}{T}$, and the tension k′ times this. The internal pressure produced by the tension is $\frac{2}{D}$ times the tension, or:

$$P_n = 2\,k'\,(1/T - 1/D) \qquad (14)$$

In exactly the same way, the internal pressure due to the perineurium may be expressed as:

$$P_p = 2\,k''\,(1/Q - 1/U) \qquad (15)$$

It is convenient to simplify the expression obtained in equation (3) for the myelin pressure by using the later equation (5). One then obtains:

$$P_m = 2\,S_c.\,t.\,\frac{1}{D} \qquad (16)$$

Using the fact that the number of nodes of Ranvier is unchanged after medullation, we have:

$$N\,L_o = G_o, \text{ and } N\,L = G \qquad (17)$$

The lengths of the nerve trunk at which the largest fibre became medullated (G_{ow}), and another fibre of diameter *D* became medullated (G_{oD}), can be related by the fact that the number of nodes on each is constant:

$$G_{ow} = N_w.\,L_o \text{ and } G = N_w.\,L_w$$

also:

$$G_{oD} = N_D.\,L_o, \text{ and } G = N_D.\,L_D,$$

Therefore:

$$G_{ow}/G_{oD} = L_D/L_w \qquad (18)$$

Using now the isometric growth postulate:

$$T = T_o.\,\frac{G}{G_{oD}} = T_o.\,\frac{L_D}{L_o} \qquad (19)$$

and similarly,

$$Q = Q_o.\,\frac{G}{G_1} = Q_o.\,\frac{G_{oD}}{G_1}\cdot\frac{L_D}{L_o} \qquad (20)$$

Finally, an expression must be found for the effective diameter *U* of the perineurium in terms of the observed lengths and diameters. According to the treatment of modality in section III, in a bimodal nerve trunk each upper mode fibre of diameter *D* will occupy a square of side *D* in a cross-section of the perineurium, so that the number of fibres belonging to the upper mode that can be accommodated is equal to $\frac{\pi U^2}{4 D^2}$, and Thomas ('54) has shown that this number of upper mode fibres does not change after the bimodality has developed, as is also implied by the theory of close packing in section (3). The effective diameter of the perineurium may therefore be expressed as:

$$U = R . D_w \qquad (21)$$

where *R* is a numerical constant related to the number of fibres in the upper mode.

The terms appearing in equation (13) have been evaluated in the subsequent three equations so that equation (13) becomes:

$$P_a = 2k\left(\frac{1}{T} - \frac{1}{D}\right) + 2k''\left(\frac{1}{Q} - \frac{1}{U}\right) + 2S_{c} . \frac{t}{D} \qquad (22)$$

The variables T, Q, and U appearing in equation (22) have been evaluated in equations (19), (20), and (21), so equation (22) becomes:

$$P_a = 2k'\left(\frac{G_{oD}}{T_o G} - \frac{1}{D}\right) + 2k''\left(\frac{G_1}{Q_o G} - \frac{1}{RD_w}\right) + 2S_{c} . \frac{t}{D} \qquad (23)$$

Equation (23) expresses the hypothesis in terms of constants and observable quantities, and may be compared with the published data. The latter consist of measurements of the largest fibre in a nerve trunk at different lengths of the nerve trunk in a growing animal, and of measurements of the diameter and internodal length of sub-maximal fibres at certain fixed lengths of the nerve trunk. In order to compare equation (23) with the data of the first kind involving the diameter of the largest fibre, we put $D = D_w$ in equation (23) and obtain:

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$$c_1 = c_2 . \frac{1}{G} + \frac{1}{D_w} \tag{24}$$

where the constants c_1 and c_2 are given by:

$$c_1 = \frac{P_a}{\left(2S_c t - 2k' - \frac{2k''}{R}\right)}$$

and

$$c_2 = \frac{\left(\frac{2k'G_{ow}}{T_{ow}} + \frac{2k''G_1}{Q_o}\right)}{\left(2S_c t - 2k' - \frac{2k''}{R}\right)}$$

For the data of the second kind, G_{ow}, L_w and D_w are constant, and by using the alternative expression for T and Q given in equations (19) and (20) and using equation (18) to express G_{oD} in terms of G_{ow}, equation (22) becomes:

$$c_3 = c_4 . \frac{1}{L} + \frac{1}{D} \tag{25}$$

where the constants c_3 and c_4 are given by:

$$c_3 = \left(P_a + \frac{2k''}{RD_w} - \frac{2k''G_1L_o}{Q_oL_wG_{ow}} \right) / (2S_c t - 2k')$$

$$c_4 = \left(\frac{k'L_o}{T_o} \right) / (S_c t - k')$$

The hypothesis that the growth of nerve fibres is limited by the mechanical constraints of the connective tissue sheaths and the myelin has been shown to lead to equations (24) and (25). These state that the diameter of the largest fibre in the nerve trunk of a growing animal will be related to the length of the nerve trunk so that their reciprocals are proportional, and that at any one fixed length of the nerve trunk the diameters and internodal lengths of the nerve fibres will also be related so that their reciprocals are proportional, with new constants of proportionality. These predictions can be tested with the published data. In the mammalian data the internodal length is usually approximately proportional to the fibre diameter (but see below). This would require $c_3 = 0$ for equation (25)

TABLE 4

Measurements by Thomas ('54) of the internodal length and fibre diameter of submaximal fibres at three different lengths of the nerve trunk, and of the diameter of the largest fibre, and the length of the nerve trunk in animals of different lengths (lateral line n. of trout)

L (μ)	$\frac{10^3}{L}$	D (μ)	$\frac{10}{D}$	LENGTH OF TRUNK *cm*
575	1.74	10.5	.952	6.3
475	2.11	8.0	1.25	
350	2.86	6.0	1.67	
300	3.33	4.0	2.50	
250	4.00	3.0	3.33	
200	5.00	2.5	4.00	
1700	0.588	19	.526	26.3
1350	0.741	14	.714	
1125	0.889	10	1.00	
850	1.18	7	1.43	
625	1.6	5	2.0	
325	3.08	3	3.33	
250	4.0	2.5	4.0	
3900	0.256	22	.455	54.5
2625	0.381	16	.625	
1575	0.635	11	.909	
750	1.33	7	1.43	
400	2.50	5	2.0	
225	4.44	3	3.33	

G (cm)	$\frac{10}{G}$	D_w (μ)	$\frac{10}{D_w}$
55	.182	32	.313
30	.333	23.1	.433
15	.667	17.2	.581
10	1.0	14.5	.69
6.5	1.54	12.0	.83
5.5	1.82	12.0	.83
5	2.0	10.3	.971
3.3	3.03	8.0	1.25
2.5	4.0	6.0	1.67
2.2	4.55	6.0	1.67
1.5	6.67	4.0	2.5

to fit such results. The results of Thomas ('54) on the lateral line nerve of the trout cover a much wider range of diameter and internodal length than is available in the mammalian data, and some of these results are given in table 4, and plotted in graphs 1 and 3, figures 3 and 4. It will be seen that in the

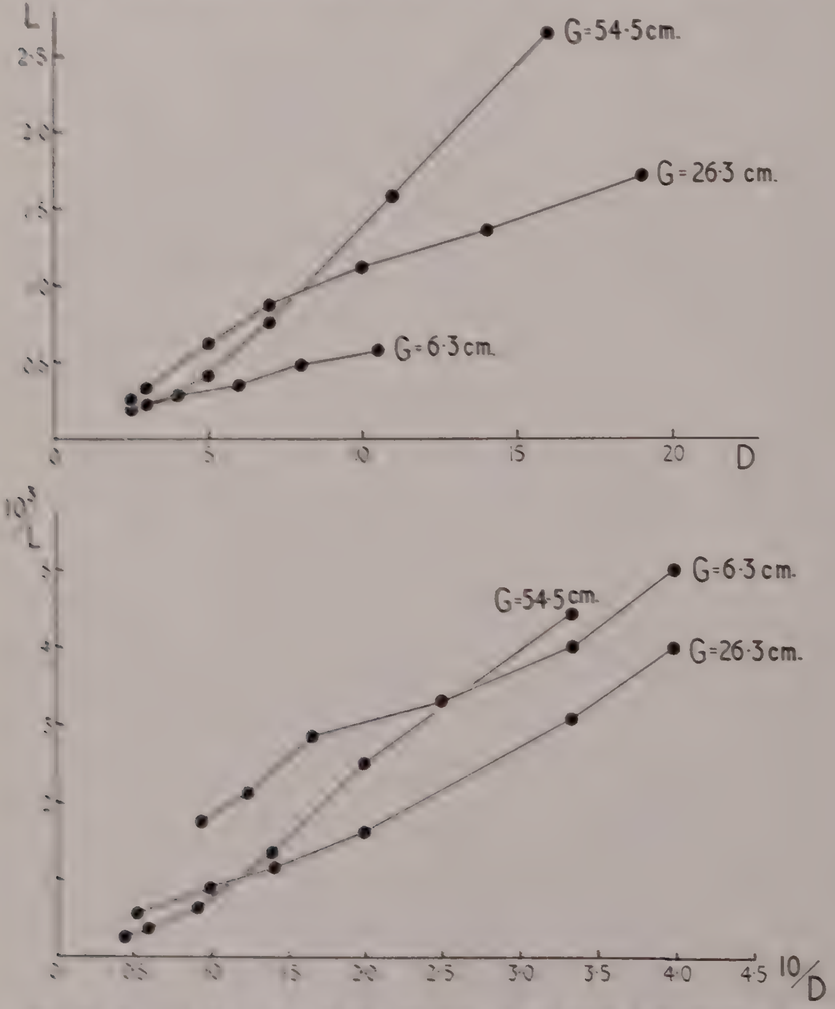

Fig. 3. Internodal length and fibre diameter in the lateral line nerves of trout of three different lengths (Thomas, '54). Graph 1 the crude data, graph 2 a plot of the reciprocals.

D × L graph (1), figure 3 the three curves for different lengths of the nerve trunk all begin at the same fundamental length and diameter, but thereafter diverge with different curvatures. A similar family of curves has been found by Thomas for the lateral line nerve of the Ray, and as this is an extensive study there is reason to think that a change of curvature with trunk

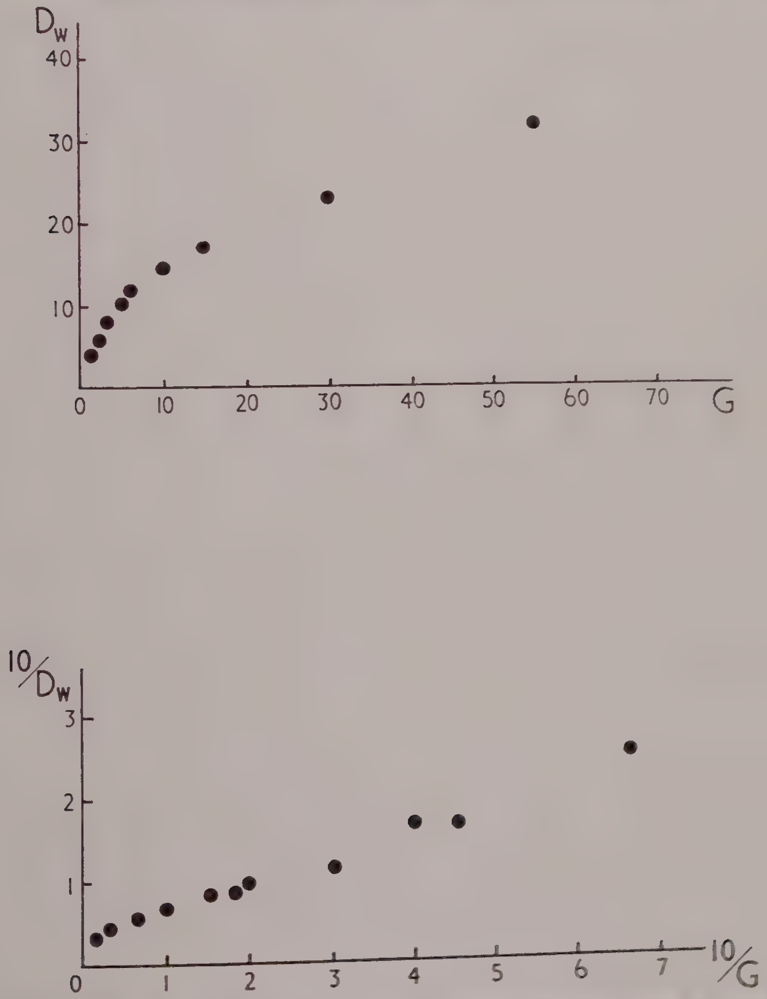

Fig. 4 The diameter of the largest fibres in the lateral line nerves of trout of different lengths (Thomas, '54). Graph 3 the crude data, graph 4 a plot of the reciprocals.

length is established. A similar effect may be seen in figure 13 of Vizoso and Young ('48), though in this narrower mammalian range no upward curvature is obtained. In graph 2, figure 3 the reciprocals of D and L are plotted from table 4, and the three curves clearly diverge very much less than the corresponding three lines in graph 1. According to equation (25) the gradients of the three lines in graph 2 depend on (l_s/T_s) and the elasticity constant k' and S_s and t, so that the gradients should be the same unless one of these factors changes during growth. The three curves will be separated, if parallel, because the constant c_s depends on the length of the nerve trunk. The three curves of graph 2 are approximately parallel except for three points on the $G = 54.5$ line. Now that there is an indication that the reciprocals of the measurements are related linearly, future work should include more measurements at the lower diameters where the reciprocals are changing most rapidly.

In graph 3, figure 4 it will be seen that the diameter of the largest fibre in the nerve trunk increases less rapidly than the length of the trunk. In graph 4, figure 4 the reciprocals plotted from table 4 are seen to be satisfactorily proportional, as required by equation (24).

The theory that the lengths and diameters of nerve fibres are related by the constraints of the myelin and connective tissue sheaths appears then to be in as good agreement with the available data as can be expected from the complicated nature of the constraints involved.

V. *Two further possible relationships.* On the basis of the hypotheses so far discussed it is possible to suggest a relation between the size of an axon and the size of its cell body, and also between the size of an axon and its rate of regeneration. There are at the moment insufficient data to test these relations satisfactorily, and they are included only as suggestions for further measurements.

The hypothesis investigated in section I suggests that there might be a continuous leakage of myelin from the axon, and a leakage over the surface of the axon has also been proposed by

Young ('44). It is likely that to a first approximation the rate of leakage would be proportional to the area of the axon. This leakage must be made good by a supply of axoplasm from the cell body, and Le Gros Clark ('32) and Young ('45) have proposed that axons of large diameter or great length have large cell bodies. There are at the moment no adequate data on the relation between the size of the cell body and the size of the axon, but Le Gros Clark ('32) (pages 322, 324, 268) has given some figures of the ranges of sizes which occur in the human nervous system. The cell bodies are said to range in diameter from 5–120 μ, and the axons from 1–20 μ. In length, axons are said to range from over two feet (say 700 mm) to very short lengths in neurones in the central nervous system (say 1 mm). It happens then that the range of cell volumes is 24^3 to 1, or 13,824 : 1, and the range of axon surface areas is 20 × 700 : 1, or 14,000 : 1. This agreement is of course fortuitous, but with the same figures, the range of axon volumes, for instance, is 20 times the range of cell-body volumes. There is some basis for expectation then that a suitable series of measurements of axon areas and cell-body volumes would show these quantities to be proportional.

The hypothesis that axons grow by an outflow of axoplasm from the cell body (Young, '45), suggests that the rate of regeneration of the axon tips should be related to the diameter of the axon and the distance from the cell body to the axon tip. The hypothesis of section IV proposed that the pressure in the cell body is determined by biochemical means, and is the same for all cell bodies. All axons would then regenerate under the same pressure difference between the cell body and the intercellular spaces, and the rate of flow of axoplasm, if it is treated as a liquid, is given by Poiseuille's equation as proportional to the fourth power of the diameter of the axon divided by the distance of the axon tip from the cell body. The quantity of axoplasm needed for the regeneration of the axon over a unit length is proportional to the cross-sectional area of the axon, or to the diameter squared. The speed of the axon tip should then be proportional to the square of the diameter of the growing axon divided by the distance of the tip from the cell-body.

There are as yet insufficient measurements to test this hypothesis, and the diameters of regenerating axon tips do not vary as much as the diameters of the matured axons. When a fibre has been crushed and is regenerating below the crush, the axoplasm is moving through tubes of different diameters above and below the crush. A formula can easily be provided for this situation, but the general result in any case is that large diameter axons should regenerate more quickly than small axons, and that the rate of regeneration should fall off with the distance of the axon tip from the cell-body. A wide range of rates of axon regeneration has been reported in the literature, ranging from several millimeters a day in the larger medullated fibres of mammals (Young, '45), to rates of the order of 100 μ a day for small fibres in the rabbit's ear (Clark, Clark and Williams, '34) and in amphibia (Williams, '30). Evans and Murray (to be published) have found a positive correlation between fibre size and rate of regeneration in the vagus nerve of the rabbit. On the other question of whether the rate of regeneration falls off with the distance from the cell-body there is a divergence of views: work on small animals such as rabbits has not detected such a deacceleration (Young, '45), but in Man evidence of deacceleration has been obtained over greater distances than are available in the ordinary experimental animals (Seddon, Medawar and Smith, '43; Sunderland, '47; Bowden and Sholl, '50). The evidence is at present inconclusive, and it would be very difficult to obtain measurements which could do more than test the hypothesis qualitatively.

DISCUSSION

Mechanical treatments of four aspects of the structure of medullated nerve fibres have been found, and they are consistent with the quantitative anatomical data. This does not preclude there being other hypotheses that might also lead to expressions consistent with the data, though the four hypotheses set out here were the only successful ones out of about a dozen that were investigated. No other hypotheses have been found which lead to even qualitative agreement with the observations. It is hoped that the theory will be useful in future anatomical investigations in suggesting which variables should be measured, and that further evidence will be forthcoming to test the validity of the results. If the success of mechanical treatments of the growth and form of the nervous system becomes established it will emphasize that biological structures grow as they do for mechanical reasons in the first place, and only secondarily because they have been selected for their functional efficiency during evolution. This point of view considerably restricts the possible theories of how the central nervous system is organised (Cragg and Temperley, '54).

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ON THE MECHANISM OF ACTIVE SODIUM TRANSPORT ACROSS THE FROG SKIN

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SIX FIGURES

It has been recognized for a long time that the electrical properties of biological membranes imply the movement or the asymmetrical distribution of ions across the surface. For many years attention was directed toward potassium ion, especially since the discovery that the distribution ratio (extracellular: intracellular) for muscle agreed closely with the ratio calculated from the potential difference (Boyle and Conway, '41). At the same time sodium ion was neglected for there was no agreement between its distribution and any electrical parameter. Only recently, with the advent of tracer studies, has intensive investigation shown that, in fact, the active transport of sodium ion is probably responsible for the generation of electrical asymmetry across such membranes as those of nerve and muscle cells (Hodgkin, '51; Steinbach, '51), and across the isolated frog skin (Ussing, '49; Linderholm, '52).

Work on the two aspects of ion movement across the frog skin proceeded independently. Direct demonstration of salt transport was reported by Huf ('35) for preparations *in vitro*, and by Krogh ('38) for intact animals. Experiments on the electrical properties of the skin were easier to carry out and

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were reported by several laboratories (cf. Greven, '41). One such experiment showed that removal of Na ions from the solution bathing the outside surface of the skin abolished the potential difference which existed when both sides were bathed by Ringer's solution (Galleotti, '04), but the significance of this observation was not appreciated until recently. In the past few years ion movement and electrical properties have been correlated, largely through the work of Teorell ('51), Ussing ('52), and Hodgkin ('51). It has been shown, for example, that the potential difference is generated by the active transport of sodium ions from the outside of the skin to the inside. There is, however, still much to be learned about the mechanism by which this transport is effected.

THEORY

Most attempts to describe quantitatively a phenomenon of active transport are developed by treating it as a special case of diffusion beginning with the general diffusion equation (cf. Teorell, '51; Ussing, '52). Few assumptions are made with this approach, and the resulting equations have led to extremely useful results. One example, which is pertinent to the work reported in this paper, was the demonstration that only sodium ion is transported actively by the isolated frog skin under normal conditions. In general, these equations find their greatest utility in distinguishing between the active and passive movement of ions; but when the approach is extended in an attempt to characterize the active transport process several shortcomings become apparent. In the first place, several quantities which appear to be parameters and which would be useful as such, turn out to be variables of the same set of forces which determine the ion movement. Two examples of such quantities are the transport force (designated E_{Na} by Ussing) and the resistance to passive diffusion of sodium (measured by the partial conductance for the ion; cf. Linderholm, '52). Thus, the equations developed by Linderholm, for example, relate the flux in one direction to the electrochemical gradient across the frog skin. But since both flux and conductance appear in the equation as a function of the gradient, little insight into the system is gained by its use. It should be stressed at the very outset that these equations are not incorrect; they do describe the transport. However, the fact that they contain two dependent variables limits their usefulness, for the environmental conditions must be specified and controlled closely in order that a change in one of the "parameters" has significance.

However, the chief limitation is the fact that little is learned about the chemical nature of the transport process by this type of study. The fact that few assumptions are made in the derivation of the equations makes them useful from a standpoint of generality, but the very generality limits their utility in providing some insight into the molecular details of the system. It is proposed to approach active transport from an altogether different direction in this paper. Certain assumptions will be made about the nature of the system. Insofar as possible these will be listed, and such justification for them as exists will be cited. It will remain to develop from these assumptions equations which will characterize the transport of sodium ions across the frog skin, and in particular to account for the following observations: (1) the nature of the rise in the rate of transport when the concentration of sodium bathing the epithelial (outside) surface is increased; (2) the appearance of a maximum in the curve relating net sodium flux to concentration; (3) the insensitivity of the net flux to changes in diffusion gradient over a large range; and (4) the nature of the change in outflux as the concentration of sodium in the *outside* solution is increased. This peculiar phenomenon was reported by Linderholm ('52), and is confirmed by the work reported here.

Assumptions:

1. *The active transport of Na ion is mediated by a "carrier" with which it forms a non ionized complex.* There is no direct experimental demonstration of such a Na-carrier. On the other hand, several investigators have invoked this type of mechanism in quite different cell types (Rosenberg,

'48; Ussing, '49; Hodgkin, '51). It is the only unitary hypothesis advanced thus far which will account both for movement against an electrochemical gradient and for the chemical specificity of the system (only Li ion provides even a poor substitute for Na).

2. *The carrier concentration is low compared with the effective concentration of the Na ion in its vicinity.*

3. *The carrier is more accessible from the outside solution than from the solution bathing the inner surface of the skin.*

4. *The complex formed at the outer surface of the skin migrates to the inner surface and releases the free ion.* This follows from the direction of the movement (from outside solution through the skin to inside solution).

5. *The rate of migration is the rate limiting process in transport.* This means that complex migration is slower than formation of the complex, and is probably to be expected.

6. *The diffusion component of a flux is proportional to the concentration of the ion in the solution from which that flux originated,* i.e. that part of the influx which is due to free diffusion is proportional to the concentration of Na in the *outside* solution. This implies a solution of the diffusion equation in which the boundary conditions are zero potential difference and constant concentration gradient. Both conditions are realized during the steady state in the experiments reported here, the latter as a close approximation. It should be stressed that the "diffusion component" is not a net movement; it is merely the number of ions moving into the skin from one of the solutions under conditions of free diffusion.

7. *Outward diffusion is in series with the sodium pump.* This means that outward moving sodium ions can interact with the carrier molecules. This is contrary to the assumption which is often made that diffusion and active transport are in parallel (cf. Linderholm, '52).

8. *The carrier cannot distinguish between ions deriving from either solution.*

Some of these assumptions are trivial in appearance, but an attempt has been made to be explicit so far as this is possible. All of them are necessary in order that the equations take the form they have. The key postulates are the first and the 7th. These two will determine the general nature of the derived relationships which are to be compared with the experimental observations.

We can write as a first approximation a reaction for the formation of the complex

$$\mathrm{C} + \mathrm{Na_o} \leftrightharpoons \mathrm{C : Na} \rightarrow \text{Transport}$$

which is really a concise statement embodying assumptions 1, 3, and 4. It will become more nearly correct as the sodium ion concentration in the external solution is increased. The equilibrium constant for the complex *decomposition* is

$$K_s = \frac{[\mathrm{C}][\mathrm{Na_o}]}{[\mathrm{CNa}]} \tag{1}$$

where $[\mathrm{Na_o}]$ is the concentration of ionic sodium in the outside solution, [C] is the concentration of free (uncomplexed) carrier molecules, and [CNa] is the concentration of the sodium-carrier complex. Since the total quantity of carrier in the preparation must exist either free or combined we can write

$$[\mathrm{C_t}] = [\mathrm{C}] + [\mathrm{CNa}] \tag{2}$$

and solving for [C] and substituting into equation 1

$$K_s = \left(\frac{[\mathrm{C_t}]}{[\mathrm{CNa}]} - 1\right)[\mathrm{Na_o}] \tag{3}$$

The rate of transport, which is dependent on the quantity of complex which has been formed, is written proportional to the complex concentration, whence it follows that the maximum transport which can be developed by the skin is proportional to the total concentration of the carrier molecules. That is

$$M_c = k[\mathrm{CNa}] \qquad\qquad M_c(\max) = k[\mathrm{C_t}]$$

where M_c is the carrier mediated influx and $M_c(\max)$ is the maximum possible carrier mediated influx. Then

$$\frac{M_c(\max)}{M_c} = \frac{[\mathrm{C_t}]}{[\mathrm{CNa}]}$$

Substituting the ratio of fluxes for the concentration ratio in equation 3 and solving for the quantity M_c

$$M_c = \frac{M_c(\text{max})[Na_o]}{K_s + [Na_o]} \tag{4}$$

The diffusion component of the influx may be written simply as proportional to the concentration of sodium in the external solution. The resistance parameters of the skin are absorbed into the proportionality constant. Thus

$$M_d = k[Na_o]$$

and the total influx, M_i, represents the sum of the carrier mediated and diffusion components.

$$M_i = M_c + M_d = \frac{M_c(\text{max})[Na_o]}{K_s + [Na_o]} + k[Na_o] \tag{5}$$

In treating the outflux of the ion the assumption was made that the diffusion path was in series with the pump. Qualitatively, assumptions 7 and 8 would effect the supposedly passive outflux as follows. When the *outside* solution contains no sodium ions the entire carrier system would exist in the free (uncomplexed) state. The outward sodium diffusion would be large up to the point where the ions come into contact with the carrier molecules. A certain proportion of the diffusing ions would encounter unoccupied carrier molecules, form a complex with them, and would be carried back into the inside solution. The rest of them would escape, and these would represent the outflux as measured, for example, with Na^{24}. Addition of some sodium ions to the outside solution would tie up some of the complexing agent, thus allowing more of the outward diffusing ions to escape. The higher the concentration of sodium in the outside solution the greater will be the fraction of the pump occupied, and the chance for collision between an outward moving ion and an unoccupied complexing molecule will be correspondingly diminished. As a result, the outflux, measured by the appearance of an isotope in the outside chamber will be increased, and the increase will be some function of the concentration of the sodium in the solution *into which* it is diffusing.

Quantitatively, the picture would be as follows. The outflux, M_o, represents the difference between the maximum number of ions diffusing into the skin and the number which are pumped back into the inside solution after collision with carrier molecules.

$$M_o = M_o(\text{max}) - M_o(\text{back}) = k[Na_i] - M_o(\text{back}) \qquad (6)$$

By reasoning analogous to that used in the derivation of equation 4 it is possible to show that the concentration of uncomplexed carrier, [C] is

$$[C] = \frac{[C_t]}{1 + [Na_o]/K_s} \qquad (7)$$

Since the diffusion into the skin depends on the concentration of sodium ion in the *inside* chamber, and since this quantity is constant (even when the concentration is varied in the outside chamber) the rate at which the outgoing sodium is pumped back can be considered to be proportional to the concentration of free carrier

$$M_o(\text{back}) = A[C] \qquad (8)$$

and combining equations 7 and 8

$$M_o(\text{back}) = \frac{A[C_t]}{1 + [Na_o]/K_s} \qquad (9)$$

Then from equations 6 and 9, the measured outflux will be given by

$$M_o = M_o(\text{max}) - \frac{A[C_t]}{1 + [Na_o]/K_s} \qquad (10)$$

The active transport of any ion actually means that a net movement of the ion is occurring in a situation where physical forces are absent. Thus, the most useful quantity in the study of this phenomenon is the net flux which is defined as the difference between the flux in one direction and that in the other; in the case of the frog skin between influx and outflux. By combining equations 5 and 9 the following expression is obtained for the net flux.

$$M(\text{net}) = M_i - M_o = \frac{M_c(\text{max})[Na_o]}{K_s + [Na_o]} + k([Na_o] - [Na_i]) + \frac{A[C_t]}{1 + [Na_o]/K_s} \qquad (11)$$

This equation can be tested by measuring the net flux as a function of variations in the concentrations of sodium in the solutions bathing either the inside or outside surface of the skin. Similarly, equation 5 can be tested by measuring the influx as a function of the external sodium ion concentration. It is to be noted that the flux described by this equation is independent of the concentration bathing the inside of the skin, and this provides another check on the validity of the assumptions used in the derivation. The outflux can also be measured while varying the external concentration and the observed data used in conjunction with equation 9.

It has probably been noted that the potential difference which exists across the skin has been neglected in this treatment, in spite of the fact that it exerts a considerable force on ions moving through the skin. The reason for this lies in the fact that all of the experiments described in this paper were run on preparations whose potential differences had been abolished by a technique which is to be described below.

METHODS

The frog skin was set up as a membrane separating two conical lucite chambers. Each chamber was connected to a small reservoir, and fluid — usually a Ringer's solution — was circulated by means of a stream of compressed air which also served for aeration. Substances added to either reservoir were thoroughly mixed in the circulating medium in less than a minute, and samples for chemical or isotopic analysis could be taken from the reservoir bathing either surface of the skin. The volume of solution bathing each surface of the skin was varied with the experimental design, but it was usually 20–25 ml.[3] The apparatus has been described by Ussing, and a schematic has been published by him ('52).

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[3] The solution bathing the epithelial (outside) surface of the skin will be referred to as the outside or external solution. The solution in the other chamber which bathes the inside surface will be called the inside solution.

The basic saline used in these experiments was a frog Ringer's solution which will be designated NaR. Its composition was as follows (millimoles per liter):

$NaCl$	KCl	$CaCl_2$	$NaHCO_3$
112 mM	2.0 mM	1.0 mM	2.5 mM

Much of this work involved a change in the concentration of sodium ion, $[Na]_s$ in one of the solutions, and this was accomplished by replacing a measured volume of the NaR with the same volume of a Ringer's solution in which choline chloride was substituted for NaCl. The composition of the choline Ringer's (CR) was:

$ChCl$	$KHCO_3$	$CaCl_2$
115 mM	2.3 mM	1.0 mM

Substitution of CR for NaR had little effect on the concentration of any ion other than sodium and left the ionic strength of the solution virtually unchanged. The latter fact was important in that changes in [Na] involved little change in its activity coefficient, and hence concentrations were used in all calculations. There was no choline flux across the skin (unpublished experiments), and it was considered to be inert in the system. Further justification for this view was found in the fact that where comparison was possible, experiments with low [Na] gave similar results in the presence or absence of choline.

A junction potential existed between a CR-agar bridge and a NaR solution, the magnitude of which was about 2 mV. In most of the experiments where choline was the predominant cation CR-agar bridges were used to minimize this potential, while if Na was the major cation NaR-agar bridges were used. Some of the experiments required that several solution changes be made during a single run, and it was not always possible to change the bridges (e.g. when the solution contained an isotope). In these cases appropriate corrections were made for the junction potential.

The electrical setup has been described by Ussing and Zerahn ('52) and equipment which is essentially the same has been used by Linderholm ('52). The skin was connected to a potentiometer through a pair of Ringer-agar bridges, and to a second external circuit through another pair of bridges. The latter circuit contained a dry cell and a variable resistance; the current flowing through this circuit could be changed by means of the latter. In the experiments described in this paper the current density across the skin was adjusted so that $IR_{skin} + E_{skin} = 0$. This was equivalent to short circuiting the skin potential through a pair of very low resistance leads, and hence the preparation will be called a short circuited frog skin, and the current flowing in the external circuit will be referred to as a short circuit current. The adjustment was made by varying the external resistance until the skin potential (measured by the potentiometer) was zero. When the skin was bathed by the same solution on both sides and the potential was set at zero, no electrochemical gradient existed for any of the ions present. Hence, under these conditions active transport was the only mechanism by which net movement could take place. Using this technique, Ussing was able to demonstrate an active transport of Na from the epithelial (outside) surface to the chorium (inside) surface of the skin.

The current flowing across the skin was measured by inserting a microammeter into the external circuit, and when the potential was set at zero the current was the maximum which could be generated by the skin. This current must have been equal to the algebraic sum of the ionic currents flowing across the preparation. These ionic currents (*net* ion movements) could be measured by using the appropriate isotopes to measure the fluxes of all of the ions in the solutions. It was shown (Ussing and Zerahn, '52) that under normal conditions Na was the only ion which showed any net movement in the short circuited preparation; that is, the current computed from the flux value agreed nearly exactly with the current measured by the ammeter. Thus, under these conditions Na is the only ion which is actively transported by the frog skin.

The experimental import of this discovery is as follows. There are two means by which active transport of ions can be measured in the short circuited skin. The net ion movements can be measured directly with isotopes, but this is relatively tedious and costly. Alternatively, the short circuit current can be measured and the net transport calculated from this value. The current, however, tells us nothing about *which* ion(s) move, nor how many of them are subject to active transport; it measures only the algebraic sum of all net transfers. Because it has been shown that the entire current is a reflection of Na transport in the preparation described, the net movement of Na can be computed from the current measurement — nothing else is contributing to the current. Obviously it is much easier to make electrical measurements than experiments with isotopes. It must be emphasized, however, that tracer experiments are necessary in "critical" experiments in order to demonstrate that the equivalence of Na transport and ammeter current holds when conditions are varied.

The current can be used to obtain a value for the net flux, but another method must be used to obtain information about the influx (M_{in}) and outflux (M_{out}). If one of these fluxes is determined directly by using a tracer, the value can be added algebraically to the net flux to get the flux in the opposite direction. Thus, in most of these experiments the outflux was measured with Na^{24} and the net movement was calculated from the current. Addition of the two gave a value for the influx, so that all three were known.

The isotopes were obtained from a commercial source. Na^{24} was used in most of the experiments and was carrier-free with an initial activity, on receipt, of about one milli-Curie per milliliter. The activity was sufficiently high that only small quantities were added to the Ringer's solution bathing the skin. Dilution of the Na in the bath was negligible even on the 4th day after receipt of the isotope (6 half lives).

When the isotope was used on the 5th and 6th day the volume of solution added diluted the NaR as much as 5%, but this caused little change in the transport parameters, and it was neglected except in the calculation of the (chemical) concentration of Na. The isotope was added to the reservoir containing Ringer's solution and about 45 minutes was allowed in order for the skin to equilibrate with the tracer. A sample was taken from this reservoir and was used as a standard, i.e. it was counted to get the specific activity of Na in the solution from which the flux originated. Duplicate 500 μl samples were taken from the other reservoir at specified time intervals (usually 30 minutes). They were dried on a hot plate and counted, and the flux was calculated.

The animals used included members of three species of European frog, *R. esculenta, R. temporaria,* and *R. oxyrhina.* Skins of all three were qualitatively similar, but large quantitative differences were noted, for example in the magnitude of Na transport. This was especially apparent when the animals were used after more than a month in captivity (they were kept in a tank in running tap water at about 15°C. during the summer and 5°C. during the winter). The most striking change occurred in the skin of *R. esculenta.* This preparation showed a large current (net Na transport) when used soon after the animal was captured. However, if it was used several months later the transport was nearly zero. The animals were not moribund, and it will be shown in a subsequent paper that chemical agents could "stimulate" the transport system in these skins to near normal activity. A preliminary note has already been published concerning the stimulation (Kirschner, '53). Skins of *R. temporaria* and *R. oxyrhina* gave larger currents even when taken from frogs which had been in the tank for 6–7 months, and for this reason these skins were used for most of the experiments reported in this paper.

RESULTS

The source of the electrical phenomena in the frog skin. It has been posited that the potential difference across the frog skin is generated by the mechanism which transports sodium ions, and it is well known that if no sodium is present in the solution bathing the outside the skin potential, usually 50–100 millivolts (inside positive), is replaced by a very small potential of about 3–5 mV in the reverse direction. The short-circuited skin displays much the same type of phenomenon; in fact, the results obtained can be taken as strong support for the hypothesis that the electrical parameters of the skin are generated by sodium transport. Thus, when the outside of the skin was exposed to CR the usual inward-directed current was replaced by a small outward-directed current, usually of the order of 3–15 microamperes. It seemed reasonable to assume that this was the result of a slow diffusion of sodium from the inside solution (115 mM/L) to the outside (sodium-free). This assumption was tested by measuring the outflux of sodium with Na^{24}. Comparison of this value with the one calculated from the ammeter current showed reasonable agreement, although the two were not always identical. A group of data given in table 1 shows that the ionic flux was sometimes higher than would have been expected from the current measurement. The deviations were small and the magnitudes involved were such that a small absolute error would result in a large percentage deviation. Huf has recently published data, however, which seems to indicate that some small potassium movement may occur under certain conditions, and this might also account for the occasional discrepancies. For our purpose the deviations which did occur are small enough to be considered within the range of experimental error, and it will be assumed that the outward directed current was derived from the diffusion of sodium, the flux originating in the inside solution.

Addition of a small quantity of Na to the outside solution caused an abrupt change in the direction and magnitude of the current. Thus, active transport was initiated by the addition of Na with no other variations, and it was therefore of interest to examine the fluxes in both directions as well as the net flux of the ion. For purposes of illustration figure

1 shows the current before and after the addition of Na to a final concentration of 1.15 mM/L (first arrow) and 5.6 mM/L (second arrow). After the initial addition the skin came into

TABLE 1

Ammeter current and sodium flux with choline-Ringer's in the external solution

SKIN	CURRENT [1] MICROAMPS	CALCULATED OUTFLUX [2]	MEASURED OUTFLUX [2]
1	—2	0.07	0.30
2	—1	0.04	0.32
3	—6	0.22	0.42
4	—3	0.11	0.09
5	—8	0.29	0.18
6	—1	0.04	0.12
7	—7	0.26	0.24
8	—13	0.48	0.26
9	—3	0.14	0.18

[1] The negative sign connotes an outward directed current.

[2] Microequivalents per hour.

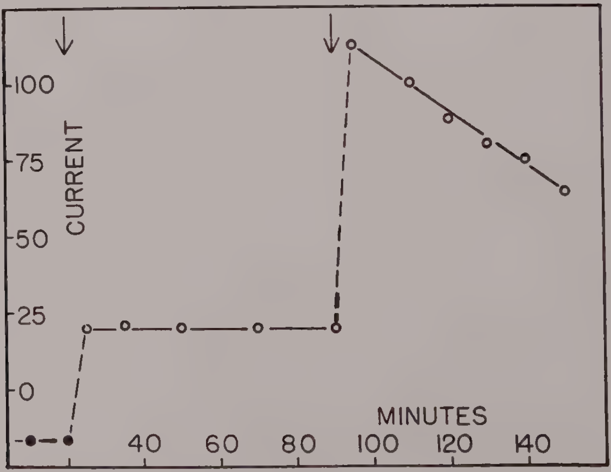

Fig. 1 Short-circuit current after addition of sodium ions to the outside of the skin. Filled circles are readings taken with Na-free choline Ringer's solution in the external chamber. The negative values indicate an outward directed current. Open circles are readings taken after the addition of Na to a final concentration of 1.15 mM/L (first arrow). The concentration was increased to 5.6 mM/L at the second arrow. The ordinate in microamperes moving across the skin. The area of the skin was 7.07 cm^2.

a steady state rapidly. The current, which had been moving outward (negative value in the figure) reversed and within 5 minutes a steady current of about 20 microamperes was measured moving inward. When the concentration was increased to 5.6 mM/L the same abrupt rise in current occurred, but in this case the current drifted downward, and the preparation was not really in a steady state at the end of an hour. The drift which was slow, occurred in most of the preparations, although usually at a higher concentration.

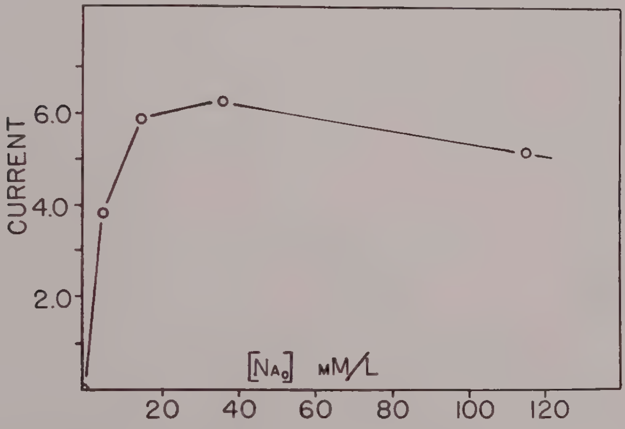

Fig. 2 Variation in short-circuit current with the concentration of sodium bathing the outside of the skin. The current was measured electrically, but it is expressed in terms of a net ion flux. The ordinate is micro equivalents per hour. Area of the skin was 7.07 cm^2.

Thus, it can be seen that the current (or the potential, if that variable is studied) is only generated when sodium is available for transport. It will be shown below that the influx of this ion increases non linearly as its concentration in the outside solution is raised. A study of the current shows that it is also a non linear function of [Na_o]. When influx and current are compared over the entire range the sodium movement is nearly always slightly higher than the net ion movement calculated from the current; that is, it is sufficient to account for the current over the entire concentration range.

The influx of sodium as a function of concentration. It was noted above that the normal potential difference exists across the skin only when the preparation is bathed on the outside with a solution containing sodium ion. It was shown long ago (cf. Linderholm, '52) that the skin potential increased with external [Na] but that it passed through a maximum and then declined slightly as the concentration was further increased. The same behavior can be shown to occur when the short circuit current is measured instead of the potential. A group of data are plotted in figure 2, and it can be seen that the current rose with increasing [Na_o], passed through a maximum at about 35 mM/L, and then decreased somewhat as the concentration was raised further. It has already been noted that the current is a reflection of the *net* sodium transport (i.e. influx minus outflux) under most conditions, and hence it might be suspected that the general shape of the curve would be determined by the influx.[4] The influx was

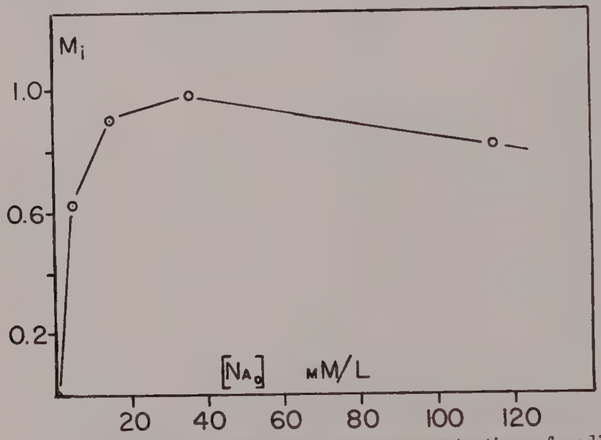

Fig. 3 Influx of sodium as a function of the concentration of sodium bathing the outside of the skin. The influx was obtained by adding the current and the outflux measured with Na^{24}. The ordinate is micromoles per square centimeter per hour.

[4] It is possible that a large rise in outflux at higher concentrations is causing the decreased net flux. As will be shown, the outflux did, in fact, increase. However, most of the increase occurred at low concentrations, i.e. before the maximum in the current-concentration curve. In addition, direct measurement of the *influx* with Na^{24} and Na^{22} showed that this variable decreased in the same manner as the net flux (or current). Hence it can be concluded that the influx does determine, at least in large part, the nature of the current-concentration relationship.

measured indirectly for the experiment shown in figure 2 by calculating the net flux from the current and adding to it the outflux measured with Na^{24} and the data[5] are shown in figure 3. It can be seen that influx rose sharply, passed through a maximum at 35 mM/L and then declined. In fact, the influx picture is virtually the same as that given by the current (which we will take as synonymous with the net sodium flux from this point on).

Inspection of equation 5 shows immediately that the maximum in the experimental curve is not predicted by theory. Thus, the first term on the right side increases with concentration, but it approaches an upper limit as $[Na_o] >> K_s$. The second term increases with concentration, and hence it could cause no decrease in M_i. In this rather critical respect the hypothesis fails and this failure will be discussed further below. Nevertheless, it should be interesting to see whether the equations can account for any of the other experimental data, for it is possible that the approach is correct but that the equations are not complete.

If the reciprocal is taken on both sides of equation 5

$$\frac{1}{M_i} = \frac{1}{M_c(\max) + kK_s + k[Na_o]}\left(\frac{K_s}{[Na_o]} + 1\right) \qquad (12)$$

The constant k includes the diffusion parameters of the skin, pore size, membrane charge and thickness, and so on, and should be the same for an ion diffusing in either direction. Therefore the value can be obtained from a measurement of the outflux through this skin. From equation 10, when $[Na_o]$ is very high

$$M_o = M_o(\max) = k[Na_i]$$

and hence when the inside solution is NaR (115 mM/L) the approximation is good, for K_s will be well under 10 mM/L. M_o for this skin under these conditions was 0.09 micromoles per square centimeter per hour and hence $k = 8 \times 10^{-7}$ L per square centimeter per hour. This value is such that the term kK_s is negligible compared with $M_c(max)$. In addition, when $[Na_o]$ is between 0–15 mM/L the term $k[Na_o]$ is also small enough to be disregarded. Thus, in the region 0–15 mM/L, which covers most of the increment in influx, equation 12 reduces to

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[5] Although the data in figure 2 were obtained by the indirect method, direct measurement of influx with Na^{24} and Na^{22} gave the same results. Either method can be used in this type of experiment with some confidence.

$$\frac{1}{M_i} = \frac{K_s}{M_c(max)} \cdot \frac{1}{[Na_o]} + \frac{1}{M_c(max)}$$

and a straight line should obtain when the reciprocal of influx is plotted against the reciprocal of the external sodium ion concentration over this range. The intercept of the line gives a value from which the maximal possible carrier activity, $M_c(max)$, can be calculated, and from the slope of the line a value can be obtained for K_s. The experimental data from figure 2 are plotted as reciprocals in figure 4 and it can be seen that over the range 5–35 mM/L the linear relationship holds reasonably well. Extrapolation of the line gives $M_c(max) = 1.16$ micromoles per square centimeter per hour and K_s was calculated to be 4.3 mM/L. It is apparent that the equation does describe the rise in influx as the concentration in the outside solution is varied over two orders of magnitude (*net* inward flux usually commences at about 0.1 mM/L).

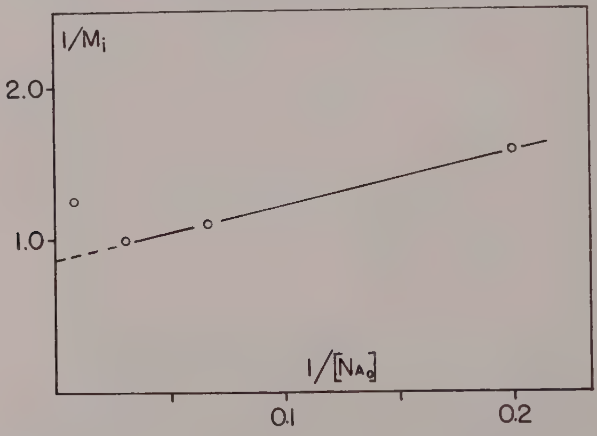

Fig. 4 Reciprocal plot of the data from figure 3. Explanation in the text.

The outflux of sodium as a function of outside concentration. It has been reported that the outflux of sodium increased when the concentration of the ion in the *outside* solution was raised from 7 mM/L to 115 mM/L. The same phenomenon is noted in the short circuited skin, and one of the purposes of this paper is to attempt to rationalize this anomalous effect. Data from a pair of experiments are presented in figure 5. They are plotted relative to the outflux with choline Ringer's (i.e. sodium-free) in the external chamber. This was done in order to get both experiments on the same scale, for the absolute fluxes across the two skins differed somewhat. It can be seen that the outflux seemed to be a hyperbolic function of the concentration, or at any rate, some function which approached an upper limit asymtotically.

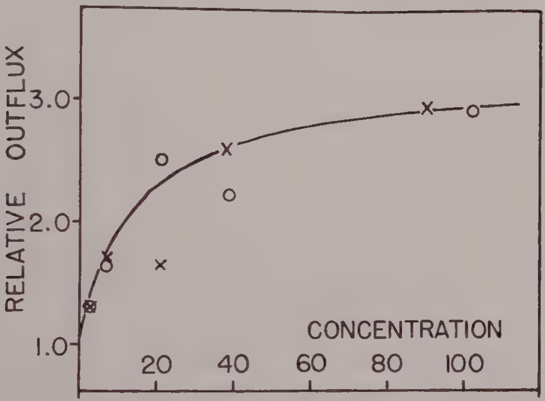

Fig. 5 Outflux of sodium as a function of the sodium concentration bathing the outside of the skin. The circles and crosses represent data from a pair of representative experiments. The ordinate is in units relative to the flux into sodium-free choline Ringer's. The absolute value of the flux with sodium-free solution was 0.045 micromoles per square centimeter per hour for one of the skins and 0.060 micromoles per square centimeter per hour for the other. The solid line was obtained by using equation 10 (cf. text).

In order to calculate a theoretical curve from equation 10 it is necessary to know the values of K_s and of M_o(max) for one of the skins. The first was evaluated by the reciprocal plot outlined in the previous section, and for this experiment it was 2.9 mM/L. M_o(max) was measured using Na^{24} as before, and its value was 0.14 micromoles per square centimeter per hour. The term $A[C_t]$ was evaluated as follows. When the outside solution is sodium-free the equation reduces to

$$M_o = M_o(\text{max}) - A[C_t]$$

and since M_o(max) is known a measurement of the outflux into sodium-free solution enables one to make the computation. For this skin $M_o = 0.06$ micromoles per square centimeter per hour under these conditions, and $A[C_t] = 0.08$ micromoles per square centimeter per hour. Since the constants are known equation 10 can be used to calculate a curve of expected outflux as a function of the outside concentration of sodium. The calculated curve is shown (solid line) in figure 5 and it can be seen that the fit with the experimental points is good. Both theoretical and experimental curves rise rapidly from a minimal value in sodium-free solution, and both approach an upper limit asymtotically.

Net flux of sodium and the diffusion gradient. The diffusion equations usually used to characterize sodium movement across the skin contain two quantities which are of interest; together they describe the force exerted on the ion. One of these is the diffusion force which is comprised of a concentration (activity) term, and the other is the term which accounts for the metabolic energy expended in moving the ion actively. The latter has been described in the theoretical section. Of the two terms describing the diffusion force the electrical potential can be neglected in these experiments, for it has been abolished in these (short-circuit) experiments. The concentration gradient (expressed as $\frac{RT}{nF}$ 1n c_o/c_i) always involves the ratio of concentrations on the two sides of the skin. This term implies that, other factors remaining constant, the net flux will increase as the concentration gradient outside : inside is increased. The influx should increase as the outside concentration is raised (inside concentration constant) and the outflux should decrease as the inside concentration is lowered (outside concentration constant). Both of these effects occur, but when the outside concentration is 10 mM/L or higher raising it further or decreasing the inside concentration leads to such a small change that in order that the diffusion equation be used it is necessary to assume that the net transport force (i.e. the metabolic term) decreases. Mathematically this is admissible, but it is just the type of behavior which limits the usefulness of the entire approach through the diffusion equations. If, for example, it were possible to determine all of the forces concerned with the movement, the equation could be used to calculate the expected fluxes. However, the force cannot be determined without using the flux measurements for the calculation, and since it varies the flux must be determined with each change in the environment in order to calculate the value of the metabolic term. Obviously, it is of no value to recalculate the fluxes by using this value.

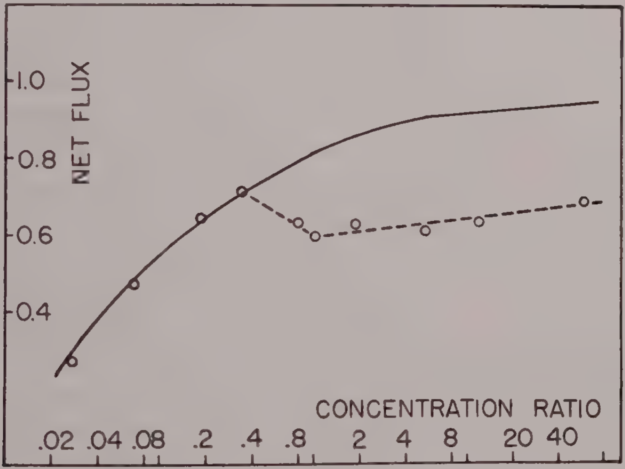

Fig. 6 Net sodium flux as a function of the concentration ratio. The first 6 points were obtained from one skin, while the last 4 were obtained from another. The abscissa shows the ratio of concentrations of sodium bathing the outside and inside of the skin, i.e. $\frac{[Na_o]}{[Na_i]}$ on a logarithmic scale. The ordinate gives the net flux in micromoles per square centimeter per hour. The solid line was calculated from theory (cf. text).

It is pertinent, therefore, to examine the *net* sodium flux over as large a range of concentration gradients as possible, and to see how closely equation 11 can predict the flux obtained. In order to extend the range, data from two different skins are used in figure 6. The gradient for the first skin was varied by setting the preparation up with 115 mM/L Na bathing the inside surface and varying [Na_o] from 1–115 mM/L. The gradient for the other skin was varied by starting with 115 mM/L on both sides and then decreasing the concentration *inside*. This particular pair of skins was chosen because the net fluxes with 115 mM/L on both sides was about the same and hence the curves could be juxtaposed without necessitating the use of relative values for the fluxes. The gradient is expressed as the concentration ratio and the abscissa is on a logarithmic scale to allow the data to be reproduced in a single figure. Values for the constants in equation 11 were obtained as in the preceding sections for one skin only; it was assumed that the values would be similar for both. The circles represent experimental points while the solid line is the theoretical curve. It can be seen that the rise in net flux is described adequately over the first order of magnitude. The "break" in the experimental separates the two curves as noted in the first section, but the virtual lack of dependence of flux on gradient is described over the last two orders of magnitude. Coincidence of the two curves over the range 2–40 could have been achieved by scaling all values relative to the data obtained with 115 mM/L on both sides. However, such manipulation of the data would add nothing to the argument; the important feature is the similarity of the curves over this region. Hence it seemed desirable to use the raw data rather than relative values.

DISCUSSION

The experiments reported in the first part of the experimental section provide very strong support for the conclusion that the electrical parameters of the *in vitro* frog skin are generated by the active inward transport of Na. When the ion is absent from the outside solution the current noted in a short circuited preparation is small and outward directed; it can largely, if not entirely, be accounted for by the outward diffusion of sodium from the Ringer's solution bathing the inside surface of the skin. Addition of Na to the external solution causes an inward directed current the magnitude of which depends on the ion concentration. Direct measurement of the influx with Na^{24}, or calculation of influx by the combination of current plus outflux measurement always showed the same thing: The influx increased as a non-linear function of added Na. Other experiments, not reported in this paper, showed that agents which increased the current (atropine) caused a comparable increase in net Na-flux (cf. Kirschner, '55), and agents which decreased the current (anticholinesterases, Kirschner, unpublished experiments; decrease in pH, Ussing and Zerahn, '51; metabolic inhibitors, Fuhrman, '52) caused a corresponding inhibition in active transport. The agreement was sufficiently good that a current calculated from the net sodium flux agreed with the short circuit (ammeter) current within the limits of experimental error.

When the skin is not short circuited and the potential, rather than the current, is studied the same electrical changes occur. The potential difference is a non-linear function of the concentration of Na bathing the outside, and the potential-concentration curve passes through a maximum (Linderholm, '52). The EMF is increased by atropine (unpublished experiments); it is decreased by low pH (Meyer and Bernfeld, '46), anticholinesterases (unpublished experiments), and inhibitors of oxidative enzymes (Huf, '35). Thus, it is apparent that the potential is closely related to the short-circuit current. The latter can be entirely accounted for by the active transport of sodium ions from a solution bathing the outside to one bathing the inside of the skin, at least in most situations.

It can be seen that the "carrier" hypothesis is successful in describing three of the experimental observations concerning the active transport of sodium ion by the *in vitro* frog skin. This appears to offer support to the hypothesis that the metabolic energy is used in operating a system in which a crucial step is the formation of a complex between the sodium ion and some mobile compound in the skin. This success is at least partially offset by the fact that the hypothesis failed completely to explain why the influx decreases when the available sodium $[Na_o]$ exceeds 20–30 mM/L. It might be of more value to discuss this failure than to attempt to extend the successful features of the equations developed at this time.

It is important to note that the maximum occurs in the influx curve. If it were present only in the curve of net flux vs. concentration the effect might be explicable in terms of the increased outflux shown in figure 5. Apparently, however, something interferes with the function of the carrier when the [Na] in its vicinity exceeds a certain value. From a physiological point of view this is perhaps not surprising for the frog is not accustomed to sitting in a solution containing 50–100 mM/L NaCl; the pump was evolved to function in pond water with a salinity of a fraction of a per cent of Ringer's solution. Such a point of view, however, does not constitute an explanation of the phenomenon.

Can the observation be rationalized within the framework of the hypothesis presented here? It can, and in fact quantitatively, but only by making one or more ad hoc assumptions. One possibility is that the carrier can combine with more than one sodium ion, but is active only when a single ion combines. Thus

$$\begin{aligned} C + Na &\leftrightharpoons CNa \rightarrow \text{Transport} \\ CNa + Na &\leftrightharpoons CNa_2 \rightarrow \text{Inactive} \\ CNa + nNa &\leftrightharpoons CNa_n \rightarrow \text{Inactive} \end{aligned}$$

An analogous situation sometimes arises in enzyme chemistry when the complex formed between enzyme and a single substrate molecule is followed by a reaction but if two substrate molecules form a complex the enzyme is reversibly inactivated. Another possibility is that the transport process initiates or involves a reaction which is autoinhibitory. At low transport rates the inhibitory process might be negligible while at higher rates it could become appreciable. Either of these hypotheses can be framed quantitatively, but since they are both ad hoc, and since no experimental evidence can be brought to bear on the problem, the specific points should not be belabored. It suffices for the present to know that the effect *can* be explained, and it should remain for further work to find the correct answer.

One thing further might be cited here in connection with the "pump failure" at high [Na_o]. A group of experiments done in an altogether different context provide considerable support for the idea that no inhibition occurs at low concentrations (it is possible, in the absence of other experimental data, that some inhibition occurs from the moment transport begins — i.e. in [Na_o] = 0.1 mM/L. This would mean that the values for M_c(max) and K_s calculated from figure 4 would be erroneous). It was noted that in one species of frog, *R. esculenta,* a marked stimulation in net sodium flux could be obtained when both sides of the skin were bathed with high Na (115 mM). This was reported in a short note (Kirschner, '53). When an attempt was made to reproduce the effect in low [Na_o] nothing happened, and an examination of the phenomenon over the entire concentration range showed that the drug was wholly without effect at low concentrations. The inference may perhaps be drawn that atropine reversed some inhibitory action, and that where it had no effect no inhibition had occurred (cf. Kirschner, '55).

While it is not possible to state that the carrier hypothesis is proved by the experiments described in this paper there is sufficient agreement between theory and observation to warrant some optimism that the approach may prove fruitful. It is certainly desirable that a new approach be made, for the diffusion equations, because of their very generality, are not likely to provide much information about the chemical nature of the active transport mechanism.

SUMMARY

A study was undertaken of some of the characteristics of the process by which the frog skin transports sodium ions

against a large electrochemical gradient. The assumption was made that such a transport required the formation of a complex between a sodium ion and a carrier molecule which was present in the membrane. On this basis equations were derived which related the influx, outflux and the net flux of the ion to the concentration bathing each side of the skin. The equations successfully described the increase in transport (net inward flux), and the increase in outflux when the ion concentrations bathing the external surface of the skin was varied over three orders of magnitude. However, the curve of influx vs. concentration passed through a maximum which was not predicted by the equation derived, and in this respect, at least, the theoretical treatment was shown to be inadequate. Some possible reasons for such a discrepancy between theory and the data were considered.

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THE EFFECT OF ATROPINE AND THE CURARES ON THE ACTIVE TRANSPORT OF SODIUM BY THE SKIN OF RANA ESCULENTA

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FOUR FIGURES

The *in vitro* frog skin has been used for extensive studies on the movement of sodium ions against an electrochemical gradient (Ussing and Zerahn, '51; Linderholm, '52; Kirschner, '55). In the course of one of these studies it was noted that when the skin of the green frog, R. esculenta, was exposed to atropine the active transport was increased in some cases to as much as 20–30 times the control level. A short note was published concerning this effect (Kirschner, '53), but a more extensive investigation was undertaken, and it is the purpose of this paper to describe the experiments which were performed.

METHODS

The preparation used in these experiments was the *in vitro* frog skin. When both sides of a skin are bathed with Ringer's solution a potential difference of 20–70 mV exists across them with the inside surface positive to the outside. It is, however, possible to drop this potential to zero by the use of a potential controlling circuit which was described originally by Ussing ('50) (cf. Ussing and Zerahn, '51). The current

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which can be drawn from this preparation is called the short circuit current and is equivalent to the sum of all of the *net* ion fluxes which occur. It has been shown that, in fact, only sodium shows a net flux under the conditions of the experiments to be described (Ussing and Zerahn, '51). Hence, the current (called the short circuit current or the ammeter current) is equivalent to the net flux of sodium ions; i.e. to the influx minus the outflux.

Most of the early experiments involved a study of the effect of "drugs" on the short-circuit current. Later the flux in one direction was measured with Na^{24} and the flux in the other direction was calculated from this measurement and the ammeter current. The critical experiments, however, were those in which the ion movements were measured in both directions simultaneously. The technique, called "double labelling" by Ussing (Ussing, '52) utilizes the pair of radioactive isotopes Na^{22} and Na^{24} and will be described in some detail.

The skin was set up as a membrane separating two conical lucite chambers. Each chamber was connected to a small reservoir but there was no communication between them except through the skin. Thus, each side of the skin could be bathed by a particular solution; substances could be added and samples taken from one side independently of the other. The solutions, usually Ringer's or a sodium-free Ringer's, were stirred vigorously by a stream of compressed air, and a substance added to one of the reservoirs was distributed uniformly through the reservoir and chamber within a minute. After addition of the Ringer's solution to each side the system was allowed to approach a steady state (time invariant current), a process which took from 30 minutes to a couple of hours.

At this time the isotope was added. Sufficient Na^{22} was added to the outside solution to give the sodium present a specific activity of 50–100 counts per minute per micromole. This was used to measure the inward movement or influx (M_i) through the skin. At the same time Na^{24} was added to the inside solution to give a specific activity of 5000–10,000 counts per minute per micromole. The Na^{24} was used to follow the outflux (M_o). About 30 minutes was allowed for the skin to equilibrate with the isotopes. Samples were taken at specified intervals from both chambers. The Na^{22} originated in the outside chamber, and hence samples taken from the inside solution were counted for Na^{22} in order to measure influx. The rate at which Na^{24} appeared in the outside solution was a measure of the outflux.

Each isotope was entering a solution which contained a very high background. Thus, the increment in Na^{22} activity in the inside chamber was usually a few counts per minute over a half hour period. This had to be counted although the Na^{24} activity of the sample taken might be of the order of 100,000 counts per minute. The reverse situation was true of outflux — the Na^{24} was counted in a sample whose background of Na^{22} was very high. A fairly simple procedure was developed which is based on the radiation characteristics of this pair of isotopes.

The half life of Na^{24} is short (14.8 hours), and it emits a fairly penetrating beta particle (1.39 MEV). Na^{22}, on the other hand, emits a softer beta (0.575 MEV), but has a three year half life. Radiation from the latter can be cut to a very few per cent by the use of an aluminum disk which will admit about 60% of the Na^{24} radiation. Duplicate 0.500 ml samples taken from the *outside* solution were dried on a hot plate and counted through the aluminum filter. They were stored for two weeks which is a period long enough for the Na^{24} activity to decay to zero. At this time they were counted again to obtain the residual (Na^{22}) activity. The difference between the first and second measurements gave the counting rate for Na^{24} and the outflux was calculated from this datum. Samples from the *inside* solution were dried and stored for two weeks. In this period the Na^{24} decayed to zero and the Na^{22} could be counted. The measurement was used for calculation of the influx. The net flux of sodium was merely the difference between influx and outflux. It is this value which, under resting conditions at least, is in nearly perfect agreement with the total *net* ion flux across the skin as measured by the ammeter current. It will be shown that even when the current varied markedly after treatment with atropine these two values agreed closely. That is, Na ion is the only one which was transported asymmetrically under these conditions.

The isotopes were obtained from a commercial source in carrier-free solution. Samples were counted with a conventional end window tube. An attempt was made to obtain at least 1000 counts for each sample and in most cases the statistical counting error was well within 3%.

It was noted early that the skins of R. esculenta showed a marked atropine effect, while skins from R. temporaria were completely unaffected by the same treatment. Because of this the experiments reported in this paper were run on R. esculenta. The animals were trapped in the spring or fall, and stored until used in a tank provided with running tap water. It was noted that animals which had been stored for longer periods showed a greater drug effect than those which were used within a few days after their capture. This will be discussed again later.

In some of the experiments it was necessary to measure transport when one of the surfaces was bathed with a solution containing little or no sodium. For these, a saline solution was used in which choline replaced sodium as the major cation. A measured volume of this solution replaced an equal volume of the regular Ringer's to give a solution containing the desired concentration of sodium ions (cf. Kirschner, '55).

For the measurement of respiration it was desirable to have a preparation which would allow the addition of drugs to one side of the skin and not to the other. The skin was stripped off the leg, and the cylinder so formed was tied off at the ankle. Between 0.5 and 1.0 cm^3 of Ringer's solution was pipetted into the bag which was then tied off at the other end. The preparation was placed in a Warburg vessel containing Ringer's solution and respiration was measured by the conventional procedure with 0.2 cm^3 10% KOH in the center well. Atropine sulfate was dissolved in Ringer's solution and the desired volume of this solution was placed in the vessel's side arm. It was tipped into the chamber at the end of the control period. In this way only the outside (epithelial) surface of the skin was exposed to the drug.

EXPERIMENTAL

1. *Experiments with sodium Ringer's solution in both chambers*

It was noted that freshly trapped frogs showed a much greater net Na-transport than did those which had remained in the tank for several weeks. The short-circuit current given by a skin from a fresh animal was frequently about 300 microamperes, corresponding to a net inward Na-flux of 1.5 micromoles per square centimeter per hour. The current from an animal that had spent some weeks in captivity was usually only 5–10% of this value, and the difference was due to a decrease in the influx of the ion, probably due to some failure of the sodium pump.

When atropine was added to the solution in the outside chamber the current increased. The magnitude of the change, absolute and relative, was larger in those skins which showed a low control value. However, the "stimulation" occurred in every preparation from frogs of the species *R. esculenta.* The data in figure 1 were obtained from a skin which had a moderately low control current. Atropine was added (first arrow) to the outside chamber to a concentration of 0.30 mM/L. When a new steady state was reached the drug concentration was increased (second arrow) to 2.2 mM/L, and a third addition made the concentration 9.0 mM/L. It can be seen that the current increased abruptly after the first two additions and rapidly reached a steady state. Equilibration was slower after the last addition; the current was still increasing slowly at the end of an hour.

Several factors might cause a change in the current. Thus, the increment might be due to a transport inward of potassium ions or by the outward migration of chloride. The former is unlikely due to the low concentration of K ion in solution, and the chloride movement seemed to be ruled out by other experiments (unpublished data). Double labelling experiments showed that, in fact, the current change could be accounted for entirely by an increased influx of sodium ions. Balance experiments were carried out, and data from two of these appear in table 1. The first group includes isotope

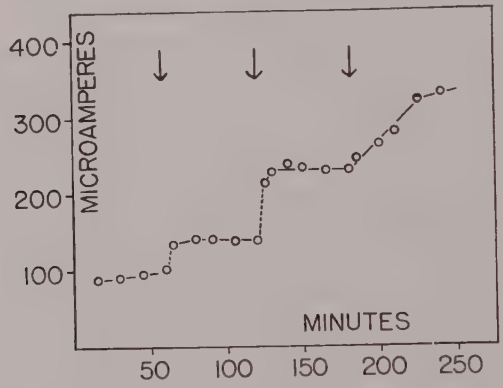

Fig. 1 The effect of atropine added to the outside of the skin. Atropine was added at the arrows (cf. text). Since the current is caused solely by net sodium movement, the net flux can be calculated from the ordinate (100 microamperes = 3.73 micromoles per hour). The area of the skin was 7.07 cm^2.

TABLE 1

The effect of atropine on sodium transport and electrical current in the frog skin

SKIN	ATROPINE CONCENTRATION	CURRENT MICRO-AMPS	CALCULATED NET FLUX MICROMOLES	INFLUX MICROMOLES	OUTFLUX MICROMOLES	MEASURED NET FLUX MICROMOLES	MEASURED / CALCULATED
	mM/L		*per hour*	*per hour*	*per hour*	*per hour*	
1.	. .	88	3.28	3.96	0.88	3.08	0.94
	. .	96	3.58	4.14	0.44	3.70	1.03
	0.3	140	5.22	5.54	0.50	5.04	0.91
	2.2	232	8.66	4.92	0.82	4.10	0.47
	9.0	313	11.67	11.58	0.82	10.76	0.92
2.	. .	80	2.98	3.61	0.66	2.95	0.99
	2.8	182	6.79	7.26	0.46	6.80	1.00
	15.0	277	10.03	13.73	2.16	11.57	1.12

and current data from the experiment described by figure 1. The fluxes were measured as described; influx with Na^{22} and outflux with Na^{24}. Current was read at 10 minute intervals on the ammeter. The current data were recalculated as a net flux (100 microamperes is equivalent to 3.73 micromoles per hour) and it can be seen that agreement between the measured and calculated net flux was very close with one exception. The agreement between electrical and ionic values for the second experiment was even closer. Hence, it can be concluded that the increase in current is caused by an increased activity of the sodium pump, and a study of the effect of atropine on this system may be performed using the simpler electrical measurements.

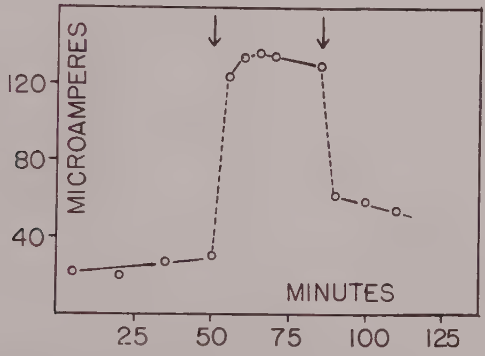

Fig. 2 Reversibility of the atropine effect. Atropine was added at the first arrow. It was washed out at the second arrow.

The atropine stimulation, at least at concentrations below 10 mM/L, is sustained for as long as the compound is in the bath. One skin maintained a 6 fold increase over the control for over 5 hours and was able to respond to more atropine administered at that time. The effect is completely reversible as is shown by the data in figure 2.

Stimulation can be effected by a pharmacologically diverse group of compounds. Data in figure 3 show the current after addition of d-tubocurarine to the outside chamber. The data are very similar to those shown in figure 1 after atropine.

Comparable effects have been elicited by histamine, pilocarpine and dihydro beta erythroidine. Acetylcholine has very little effect in concentrations up to 10 mM/L and tetra ethyl ammonium ion was without effect in one experiment.

Addition of atropine or d-tubocurarine to the solution bathing the *inside* of the skin had a different effect. There was a transient increase in the current but the rise was not maintained. Figure 4 shows the current after addition of d-tubocurarine to the inside of the skin. This effect has not been investigated further.

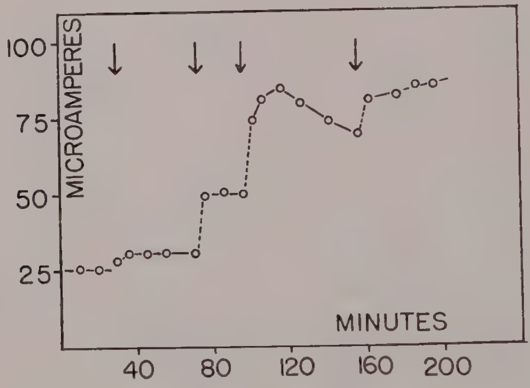

Fig. 3 The effect of d-tubocurarine on the outside of the skin. Four additions were made, one at each arrow. The curare concentrations were 0.13 mM/L (first arrow), 0.63 mM/L (second arrow), 1.3 mM/L (third arrow), and 1.9 mM/L (fourth arrow).

2. *Experiments with low sodium outside*

It was shown in a previous paper that the skin can generate more current when the outside of the skin is bathed with a solution containing less Na than 30 mM/L (i.e. 0.3 Ringer's solution). Some evidence seemed to indicate that an autoinhibitory process occurred at the higher concentrations, and it seemed that atropine might prove useful in testing this idea. The reasoning went as follows. The rise in sodium transport reported above might be a real "stimulation," but it could equally likely reflect the reversal of some inhibitory process. Since the evidence indicated that "inhibition" occurred at high but not at low (outside) sodium concentrations the drug should be ineffective in sodium concentrations below 30 mM/L, or wherever the inhibition began.[3] It was necessary, therefore, to test the drug on skins which were bathed by low Na-solutions on one side. The skin was set up with regular Ringer's solution bathing the inside surface and choline Ringer's, which is sodium-free, bathing the outside.

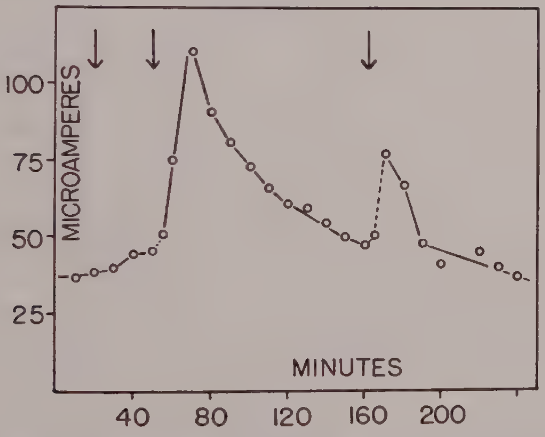

Fig. 4 The effect of d-tubocurarine on the inside of the skin. Three additions were made to the solution bathing the inside surface of the skin. The concentrations were 0.13 mM/L (first arrow), 0.80 mM/L (second arrow), and 3.0 mM/L (third arrow).

A small quantity of NaR was added to the outside chamber, usually enough to make the sodium ion concentration about 0.5 millimolar. This initiated an inward transport which could be measured by the appearance of an inward directed current (cf. Kirschner, '55). The Na was then washed out of solution and the skin was reequilibrated with C.R. Atropine was added to the outside chamber and after a few minutes incubation the same quantity of Na was added as in the control run. Table 2 shows the results of a pair of experiments. It can be seen that the compound had no effect on the transport of sodium initiated by adding a small quantity of the ion to the outside solution. Neither did it modify the outward diffusion when the external chamber contained a sodium-free solution.

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[3] The experiments in the previous paper employed skins from another species of frog, *R. temporaria*. The "inhibition" in *R. esculenta* actually occurs at lower sodium concentrations; about 15–20 mM.

3. *Experiments on respiration*

It seemed of interest to see whether these drugs stimulated the current by modifying energy turnover. Respiration of the skins is easy to measure in the Warburg apparatus, but

TABLE 2

The effect of atropine on current across the frog skin

SKIN	TREATMENT	DRUG CONC.	CURRENT [1,2]	
			Sodium-free	Low sodium
		mM/L	*microamperes*	
1.	Control	. .	— 11	+ 17
	Atropine	7.0	— 10	+ 14
	Control	. .	— 11	+ 17
2.	Control	. .	— 11	+ 17
	Atropine	4.1	— 10	+ 12
	Control	. .	— 11	+ 12

[1] Negative current is outward directed; positive is moving inward.

[2] Ten microamps is equivalent to a net flux of 0.37 micromoles per hour.

TABLE 3

The effect of atropine on the oxygen consumption of frog skins

SKIN	ATROPINE	RESPIRATION [1]	
		Control	Treated
	mM/L		
1.	3.8	7.0	6.0
2.	3.8	9.1	8.0
3.	2.1	3.5	3.7
4.	2.1	5.1	4.6
5.	6.2	5.3	5.7
6.	5.1	4.9	5.0

[1] Average values for periods of at least one hour. The values are microliters per square centimeter skin per hour.

it was necessary to use a preparation in which the drug could be added to the outside. For these reasons the skin bags described in the section on methods were used. The oxygen consumption was measured for an hour and the atropine was tipped in from a side arm at this time. The results of several experiments are shown in table 3, and it can be seen that there was no consistent change in respiration following the addition of the drug.

DISCUSSION

These experiments show that certain drugs are able to increase the net transport of sodium ions when they are added to the outside of the skin of R. esculenta. It must be stressed that the phenomenon is not general; in fact, it has been demonstrated unequivocally only with this species. Skins from *R. oxyrhina* show a small effect while those of *R. temporaria* are completely refractory. Nevertheless, the response is so great when it does occur that it appears worthwhile to study it in some detail. A study is being undertaken now to see whether any of the species found in the U. S. show this drug effect.

Little can be said regarding the chemical events which cause the rise in transport. The pharmacological diversity of the drugs used seem to argue against any simple competition with acetylcholine such as is shown by atropine on the heart and the curares on skeletal muscle. Thus, pilocarpine is not a competitor at all; instead it is parasympathomimetic in action. Histamine has no demonstrable connection with acetylcholine activity — certainly none which would cause it to be classed together with the other compounds. Perhaps most striking, however, is the relative inactivity of acetylcholine itself. If atropine acted by competing with intracellular acetylcholine it might be expected that the latter would reverse the atropine effect. In fact, no such thing occurs; acetylcholine is nearly inactive, perhaps slightly stimulatory at high concentrations, on this preparation. It is frequently argued that lack of an effect by acetylcholine may be due to the fact that it is unable to penetrate into the cell (Rothenberg, '48). It is probable that this compound does not penetrate into the frog skin although there is no conclusive evidence for this. However, d-tubocurarine, a much larger, divalent cation exerts an effect on the transport system, and hence the argument based on charge is less convincing for this preparation than for others (e.g. nerve).

In connection with the drug action, an experiment reported by Barnes ('39) might be of interest. This investigator showed that the potential difference across the frog skin was increased about three fold by the dye, eosin. It is, of course, not known whether this has any relationship to the "atropine effect." However, it is well known that at least one dye, methylene blue, has an atropine-like action on the heart (Cook, '26) and it might be that eosin is acting in the same manner.

It was shown in a previous paper (Kirschner, '55) that sodium transport increased with the quantity of the ion presented to the pump (i.e. with the concentration bathing the outside of the skin). However, when the concentration was made higher than about 30 mM/L the pump activity decreased and the idea was tentatively offered that this might be due to some kind of autoinhibition which occurs at high rates of transport. The atropine effect, while giving no conclusive answer, seems to fit some such picture. The drug is wholly ineffective in the region where the pump activity is not inhibited, but increases the transport rate markedly over the region in which the apparent autoinhibition has occurred. It might be profitable therefore to consider that this apparent stimulation is really due to the reversal of an inhibition imposed by the experimental set up. In any event, the mechanism by which these compounds exert their effect is an interesting problem the solution of which should cast some light on the molecular nature of the transport process itself.

Another puzzling observation concerns the apparent species specificity of the effect.

Several other problems remain unsolved. Perhaps the most puzzling is the reason that the atropine effect can be elicited from the outside of the skin while another, quite different picture is seen when the drugs are added to the inside. Mention was made in a previous paper (Kirschner, '55) that transport of the ion may depend on the formation of a complex with some compound formed by the skin. Orientation of the transport process indicates that complex formation and perhaps synthesis of the complexing agent would occur at the outer surface of one of the membranes involved (i.e. cellular or basilar). It might be that the drug effect reported here is due to relief of the inhibition of such a process. This would explain why the locus of action is on the epithelial surface. No explanation can be offered at this time for the rise and fall of the current when the drug was added to the inside surface, and it is not even known whether this is entirely due to changes in sodium movement (no double labelling experiments were carried out). Another unexplained phenomenon is the specificity of the drug-induced transport increase. The most striking change was seen in skins of the species *R. esculenta*. The toad *B. bufo* was also sensitive to these agents, and to a much lesser extent so were preparations from *R. oxyrhina*. However, skins from the species *R. temporaria* were entirely resistant to the drugs. A third problem relates to the diminution in Na-transport through skins of *R. esculenta* when the animals are stored prior to use. The failure does not seem to be one of energy turnover if oxygen consumption is taken as the criterion. There is little or no change in oxygen consumption under these conditions. In addition, the transport can be returned to a near "normal" level by the experimental treatment reported here with no significant increase in Q_{O_2}. It is probable that the solution of any one of these questions will afford more insight into the chemical nature of the sodium pump.

SUMMARY

It was found that a series of pharmacologically diverse drugs increased the active transport of sodium ions by the frog skin as much as an order of magnitude. The effect, noted only when the compound was added to a Ringer's solution bathing the outside surface of the skin, was sustained as long as the drug was present; it was completely reversible. Among the effective agents were histamine, atropine, d-tubocurarine and pilocarpine. Acetylcholine was ineffective.

The drug-induced increase in ion movement was seen only when the outside of the skin was bathed with a high concentration of sodium ions (above 30 mM/L). When most of the sodium was replaced by choline the drugs were ineffective.

The oxygen consumption of the skins was unaffected by the drugs.

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THE ANTICOAGULANT EFFECT OF ADENOSINETRIPHOSPHATE (ATP) ON BLOOD PLASMA [1]

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FOUR FIGURES

INTRODUCTION

Protoplasm and blood have been shown by Heilbrunn ('28) to possess comparable mechanisms for coagulation, i.e. the surface precipitation reaction and the gelation of protoplasm are both closely related to the clotting reaction of blood. Heilbrunn ('40) reported the similarity in the physicochemical behavior of the protoplasm of muscle to that of other cells. The clotting activities of blood plasma, muscle protoplasm, and protoplasm in general were discussed by Heilbrunn ('43) and were shown to adhere to the same general pattern; namely, that upon excitation or injury of a cell, thromboplastic substance(s) are released which in the presence of ionic calcium bring about a gelation or clotting of both protoplasm and blood plasma.

In 1941, Banga and Szent-Györgyi, working with the skeletal muscle of the rabbit, isolated two types of protein which more recently have become known as myosin and actin. These proteins, which form a complex (actomyosin) are thought to be constituents of the contractile elements of muscle fibres. Szent-Györgyi ('51) postulated that ATP participated in muscular contraction by acting on actomyosin. He claimed further that both cardiac muscle and smooth muscle contained contractile systems similar to that found in skeletal muscle. Contrary to this, Folkow ('49) has found that ATP acts as a vasodilator probably through relaxation of the smooth muscle surrounding the blood vessel. In this laboratory, furthermore, it has been found that ATP has the ability to relax cardiac muscle.

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The colloid theory of protoplasmic stimulation (Heilbrunn, '43), maintains that the protoplasmic gelation evidenced in muscular contraction and the gelation involved in the coagulation of blood are aspects of the same biological phenomenon. A study of the effects of ATP on the blood clotting mechanism, therefore, should be of value in elucidating the colloidal phenomenon involved in the contraction of muscle.

MATERIALS

Plasma: *(a) Oxalated duck plasma* — 9.0 cm^3 of venous blood drawn from the wing or leg into a syringe rinsed with and and containing 1.0 cm^3 of 0.1 M sodium oxalate. The blood was centrifuged for 20 minutes at 2000 RPM after which the clear supernatant plasma was removed with a pipette and placed in a test tube kept in an ice and water bath. Plasma was freshly prepared for each experiment unless otherwise stated.

(b) Heparinized duck plasma — prepared as above except that 1.0 cm^3 of heparin solution (1.3 mg per cubic centimeter of 0.85% saline solution) was substituted for the sodium oxalate. The heparin was manufactured by Hynson, Wescott, and Dunning, Lot no. 190.

Thromboplastin: prepared from acetone defatted and dehydrated duck brain. Suspensions were made in saline solution according to the method of Rosenfield and Tuft ('47).

Thrombin solution: prepared from oxalated duck plasma according to the method of Eagle ('35).

Saline solution: 0.85% sodium chloride in distilled water.

Adenosinetriphosphate (ATP): $Na_4ATP \cdot 3H_2O$ prepared and guaranteed by Rohm and Haas, Philadelphia, Pa., to be at least 95% pure, provided the salt is kept from hydrolyzing. The dry salt (in a screw cap vial) was kept in a desiccator over calcium chloride in a refrigerator. Solutions were either used on the day that they were prepared or kept frozen for use on the next day, the solvent being 0.85% saline solution. In no case was any ATP solution used more than 36 hours after preparation. The concentrations of all these solutions were checked by labile phosphate determinations according to Hawk, Oser and Summerson ('47). Of 21 such solutions, all but 4 assayed at 100% or more, the average being 104%. During the course of an experiment, the ATP solution was always kept in an ice and water bath unless otherwise stated. The pHs of these solutions were approximately 7.0.

Adenylic acid (muscle): Adenosine-5-PO_4 prepared by Nutritional Biochemicals Co., Cleveland, Ohio. A fresh concentrated solution of adenylic acid was made up for each experiment by dissolving the acid in Na-diethylbarbiturate buffer at pH 7.36. All dilutions of this solution were made with saline solution and were kept in the ice and water bath while being used.

ATP-ase: prepared as myosin with ATP-ase activity from frog muscle according to Bailey ('42). The enzyme preparation was kept in the ice and water bath unless otherwise specified. Preparations more than 48 hours old, even though frozen, were discarded.

EXPERIMENTAL RESULTS

Preliminary experiments were made on a clotting system consisting of oxalated duck plasma plus a thromboplastin-calcium chloride mixture. It was found that the addition of an ATP solution to such a system inhibited rather than accelerated the clotting reaction. These preliminary experiments were repeated on heparinized plasma plus thromboplastin (with no added calcium chloride) and again ATP inhibited the reaction to a similar degree. Since ATP had an effect opposite to that expected from Szent-Györgyi's work, it is of great interest to determine how such an action could occur and if possible the mechanism involved.

In the oxalated system, even if ATP were not present, thromboplastin in the absence of ionic calcium would not be expected to promote clotting. In the heparinized system, however, the original plasma calcium was unaltered and thromboplastin should have restored the clotting ability, providing the ATP had had no effect on calcium. The fact that the heparinized plasma did not clot under these conditions, indicates that the anticoagulant action of ATP was perhaps due to the decalcification of the plasma via the formation of an insoluble calcium ATP. The precipitation of such a compound was described by Fiske and SubbaRow ('29).

If the action of the ATP was merely calcium removal, the addition of more calcium to the system should restore the normal clotting activity. Plasma clotting systems were set up containing either an ATP solution or an equivalent volume of saline solution instead. These systems were tested against concentrations of calcium chloride varying from 0.00 M (where an equivalent volume of saline solution was used) to 0.10 M. The method used was similar to the Rosenfield and Tuft (loc. cit.) modification of the original Quick ('38) method. The thromboplastin suspension and calcium chloride solutions were kept in a constant temperature water bath at 38°C. while the plasma, saline solution, and ATP solution were kept in an ice and water bath at about 5°C. Each clotting reaction was carried out as follows:

A clotting tube containing 0.1 cm^3 of oxalated plasma plus 0.1 cm^3 of saline solution plus 0.1 cm^3 of thromboplastin was incubated for 30 seconds in the water bath at 38°C. after which the tube was removed and placed in a rack at room temperature.

To this, 0.1 cm^3 of the calcium chloride concentration being tested at that time was added to the clotting tube mixture and a stop watch started at the same instant.

The tube was returned to the water bath (usually within 6 seconds of the addition of the calcium chloride solution) and gently tilted backward and forward at a uniform rate until the clot formation started at which time the stop watch was stopped and the clotting time noted.

For each concentration of calcium chloride used, three such determinations were made. Parallel clotting reactions were done using 0.1 cm^3 of 0.01 M ATP in place of the 0.1 cm^3 of saline solution. The data presented in table 1 show that the additional calcium chloride can restore the clotting activity to plasma already inhibited by the presence of ATP. However, the normal level of clotting activity is not restored.

TABLE 1

The effect of ATP on the clotting of oxalated duck plasma in the presence of varying concentrations of calcium chloride

CONC. OF $CaCl_2$	AV. CLOTTING TIME OF PLASMA PLUS SALINE SOLUTION	AV. CLOTTING TIME OF PLASMA PLUS ATP SOLUTION
	sec.	*sec.*
0.000 M	No clot within 30 min.	No clot within 30 min.
0.025	17.3	41.1
0.040	19.7	37.1
0.060	20.3	37.1
0.080	23.6	42.6
0.100	27.9	50.8

Oxalated plasma represents a decalcified system, but heparinized plasma represents a system in which plasma calcium is present in its normal concentration. By applying ATP to heparinized plasma, its effect on a more nearly normal (with respect to calcium) clotting system can be studied. The experimental determinations were made in a manner similar to those performed on oxalated plasma. Steps had to be taken, however, to avoid any premature clotting that might occur if heparinized plasma and thromboplastin were incubated together. Consequently, each calcium chloride solution was mixed with an equivalent volume of thromboplastin suspension so that these two constituents were thus added simultaneously whenever used. These mixtures were kept in the constant temperature water bath at all times. Briefly, then, the clotting reaction for heparinized plasma was carried out as follows:

A clotting tube containing 0.1 cm³ of heparinized plasma plus 0.1 cm³ of saline solution was incubated for 30 seconds at 38°C. in a constant temperature water bath, after which it was removed and placed in a rack at room temperature.

TABLE 2

The effect of ATP on the clotting of heparinized duck plasma in the presence of varying concentrations of calcium chloride

CONC. OF $CaCl_2$	AV. CLOTTING TIME OF PLASMA PLUS SALINE SOLUTION	AV. CLOTTING TIME OF PLASMA PLUS ATP SOLUTION
	sec.	*sec.*
0.000 M	43.3	No clot within 30 min.
0.025	28.5	55.0
0.040	35.0	61.5
0.060	35.2	58.6
0.080	35.1	65.8
0.100	40.8	92.5

A 0.2 cm³ portion of one of the calcium chloride-thromboplastin mixtures was added and the stop watch was started at the same instant, the clotting time being taken, as before, while tilting the tube backward and forward in the water bath. Three determinations were made for each concentration of calcium chloride in the range from 0.00 M (where saline solution was used) to 0.10 M. Three determinations were also made for each concentration, substituting 0.01 M ATP solution for the saline solution. The results for this experiment are given in table 2 and show that heparinized plasma behaves in a fashion similar to that of oxalated plasma with respect to ATP, despite the fact that the former had its original plasma calcium unaltered while the latter had no free calcium present. In both of these systems, it can also be shown that an increase in calcium concentration accelerates clot formation while an excess inhibits it.

It is obvious that the clotting activity of the ATP treated plasma can be restored by additional calcium. The normal clotting time of such plasma, however, can not be. This suggests that perhaps the ATP acts on some other component of the clotting system. Some of the other components that could be affected are Ac-globulin, prothrombin, thromboplastin, thrombin, and fibrinogen. ATP could thus inhibit the conversion of prothrombin to thrombin by inactivating either Ac-globulin, prothrombin, or thromboplastin. It is also possible that ATP could prevent the conversion of fibrinogen to fibrin by inactivating the thrombin or fibrinogen or both.

To determine whether the ATP had any effect on thromboplastin, various tests were used. The first of these consisted of determining whether prolonged contact with ATP would affect the clot promoting ability of thromboplastin. The experiment was conducted as follows:

A reaction tube containing 2.0 cm^3 of thromboplastin suspension plus 2.0 cm^3 of saline solution was placed in the constant temperature bath at 38°C. At various intervals from zero time to 180 minutes, 0.2 cm^3 aliquots were removed and added to a clotting tube containing 0.1 cm^3 of plasma which had been previously incubated in the constant temperature bath for 30 seconds. At this time, 0.1 cm^3 of 0.025 M calcium chloride solution was added, the stop watch started, and the clotting time taken as usual.

Three such determinations were made for each time interval. Another reaction tube containing 2.0 cm^3 of thromboplastin suspension plus 2.0 cm^3 of 0.01 M ATP solution was placed in the water bath and clotting times were taken at intervals equal to those for the thromboplastin-saline reaction mixture. The data are reported in table 3 and from them it is apparent that the ATP had a considerable inhibitory effect on thromboplastin activity.

Since the inhibitory action of the ATP increases with time, two routes for progressive inhibition present themselves. Either the ATP inactivates increasing amounts of thromboplastin; or there is a progressive hydrolysis of ATP, and the breakdown products act on thromboplastin. To test this possibility, the reaction was carried out in the constant temperature water bath at 38°C. for three hours in two different ways as follows:

One reaction tube contained 2.0 cm^3 of thromboplastin suspension plus 2.0 cm^3 of 0.025 M calcium chloride solution. At the same time, 2.0 cm^3 of 0.009 M ATP solution was incubated by itself in a separate tube in the same water bath. At specified intervals, 0.1 cm^3 of chilled oxalated plasma was placed in a clotting tube and incubated for 30 seconds after which 0.1 cm^3 of ATP solution was added followed by 0.2 cm^3 of thromboplastin-calcium mixture. The stop watch was started and the clotting time taken in the usual way. The other reaction tube contained 2.0 cm^3 each of 0.009 M ATP and thromboplastin. At the same time, 2.0 cm^3 of 0.025 M calcium chloride solution were incubated in the same water bath but in a separate tube. Plasma samples were incubated for 30 seconds after which 0.1 cm^3 of calcium chloride solution was added followed by 0.2 cm^3 of thromboplastin-ATP mixture. The stop watch was started and the clotting times noted.

TABLE 3

The effect of incubating thromboplastin with ATP on the clot promoting ability of thromboplastin on oxalated duck plasma

INCUBATION TIME	AV. CLOTTING TIME WITH THROMBOPLASTIN PLUS SALINE SOL.	AV. CLOTTING TIME WITH THROMBOPLASTIN PLUS ATP SOL.
min.	*sec.*	*sec.*
0	15.8	33.1
30	19.7	67.7
60	27.6	98.4
120	33.1	130.1
180	31.9	159.3

Three determinations were taken for each interval of time and the averages reported in table 4. As can be seen from these data, the progressively inhibitory effect of the ATP was not primarily due to its gradual hydrolysis during incubation. It was more probably due to an increasingly greater combination with or inactivation of the thromboplastin by the ATP brought about while these two substances were in intimate contact during the three-hour reaction period. The fact that thromboplastin and ATP do form some sort of a bond will be brought out by another experiment later on.

In the preceding experiment, evidence was presented which showed that ATP was probably capable of inactivating thromboplastin. Such inactivation was manifested experimentally by the inhibitory effect ATP had on plasma clotting. This effect is directly attributable to the action of ATP on thromboplastin. Despite this inhibition, plasma did show clot formation but not at the normal rate. It is possible that, with the concentrations of thromboplastin and ATP used, a substantial quantity of thromboplastin still remained active. It was this free thromboplastin, then, which probably participated in the coagulation process. Therefore, it can be postulated that with lesser concentrations of thromboplastin ATP should produce greater inhibitory effects. Conversely, with the more concentrated thromboplastin suspensions, the

TABLE 4

A comparison of the effect of ATP on thromboplastin when the method of incubation is varied

INCUBATION TIME	AV. CLOTTING TIME WHEN THROMBOPLASTIN AND ATP ARE INCUBATED SEPARATELY	AV. CLOTTING TIME WHEN THROMBOPLASTIN AND ATP ARE INCUBATED TOGETHER
min.	*sec.*	*sec.*
0	31.1	55.6
30	34.1	93.0
60	36.8	123.3
120	38.9	188.5
180	40.6	200.5

inhibitory action of ATP should become less pronounced. An experiment to test this postulate was conducted as follows:

A thromboplastin suspension was prepared in the usual way. Such a suspension was considered as a 100% suspension. A series of dilutions (v/v) of this suspension was prepared using saline solution as a diluent. The series ranged from 0% (where saline solution was used in place of the thromboplastin suspension) to 100%. Sufficient 0.025 M calcium chloride was added to each dilution of thromboplastin in a ratio of 1:1 by volume. Each thromboplastin-calcium chloride mixture was kept in the constant temperature water bath at 38°C. A clotting tube containing 0.1 cm^3 of oxalated duck plasma plus 0.1 cm^3 of saline solution was incubated for 30 seconds in a constant temperature water bath. The tube was removed, 0.2 cm^3 of the respective thromboplastin-calcium chloride mixture was added and the clotting time was taken in the usual way.

Three determinations were made for each concentration of thromboplastin, and the clotting times were averaged. For each concentration of thromboplastin, three additional determinations using ATP were made. In these latter determinations 0.1 cm^3 of 0.011 M ATP was substituted for 0.1 cm^3 of saline solution. The data are plotted in figure 1.

It can be seen that the inhibitory effect of ATP increased as more dilute thromboplastin suspensions were used. In other words as thromboplastin becomes more dilute, ATP is able to bind a greater proportion of it. Furthermore, the slopes of the clotting time curves for thromboplastin plus ATP and for thromboplastin plus saline solution are very similar from 100% to about 75% concentration of thromboplastin. Below 75%, however, the curves become increasingly divergent. This divergency is primarily due to the steeper slope of the thromboplastin plus ATP curve. This shows that above 75% concentration of thromboplastin an increase in this concentration does not appreciably reduce the inhibitory effect of ATP. Thus, it may be inferred that at least 25% of a 100% thromboplastin suspension remains unbound upon first being reacted with 0.01 M ATP. Of course, with time, probably less and less thromboplastin remains free.

It is conceivable the effect was due not to an interaction between ATP and thromboplastin but rather to decalcification. If the latter were true, then the additional calcium in the system should alter the critical percentage obtained in the preceding experiment where 0.025 M calcium chloride was used. Consequently, the experiment was repeated in its entirety using 0.045 M calcium chloride instead. This concentration was now sufficient to restore all the calcium that could possibly have been removed by 0.011 M ATP plus 0.01 M

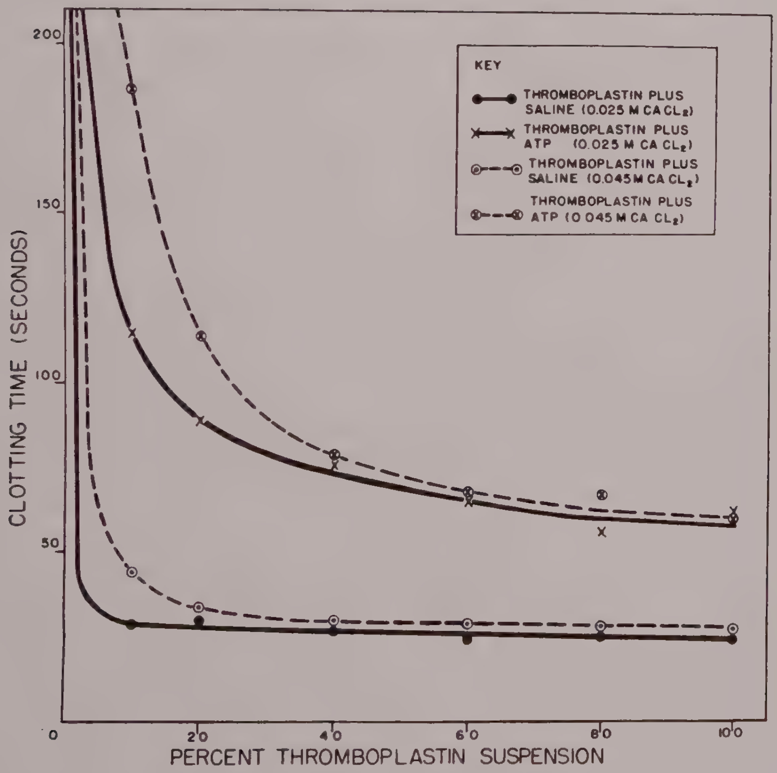

Fig. 1 The effect of ATP on various dilutions of thromboplastin (using 0.025 M and 0.045 M $CaCl_2$).

sodium oxalate and still leave sufficient calcium to bring about normal clotting. These results are also plotted in figure 1 and show that excess calcium had no effect other than to inhibit coagulation.

To demonstrate further that the previously postulated bond between thromboplastin and ATP exists, the effect of the former on the enzymatic hydrolysis of ATP was studied. If the bond exists, the effects of such a binding should show up as an inhibition of the ATP hydrolysis rate. Phosphate release by ATP-ase was measured colorimetrically according to the method of Fiske and SubbaRow ('25). In order to reduce the color intensity in these tests, more dilute than usual solutions of ATP (0.0052 M) and thromboplastin suspension (30%) were used.

Five reaction tubes were set up in the constant temperature water bath 38°C. Each contained 1.5 cm^3 of sodium diethylbarbiturate buffer at pH 7.36 plus 1.5 cm^3 of 0.00125 M calcium chloride. In addition to these two, other constituents were added. The first tube, measuring the rate of the hydrolysis of ATP by ATP-ase in the presence of calcium chloride contained 1.5 cm^3 of ATP solution, 1.5 cm^3 of ATP-ase, and 1.5 cm^3 of saline solution. The second tube, measuring the possible phosphate release from thromboplastin alone, contained 1.5 cm^3 of this component plus 3.0 cm^3 of saline solution. The third tube, measuring the possible phosphate release from thromboplastin by ATP-ase, contained 1.5 cm^3 of thromboplastin suspension, 1.5 cm^3 of ATP-ase, plus 1.5 cm^3 of saline solution. The 4th tube, measuring the effect of thromboplastin on ATP, contained 1.5 cm^3 of ATP solution, 1.5 cm^3 of thromboplastin suspension, plus 1.5 cm^3 of saline solution. The 5th tube, measuring the effect of thromboplastin on phosphate release from ATP by ATP-ase, contained 1.5 cm^3 of ATP solution, 1.5 cm^3 of thromboplastin suspension, plus 1.5 cm^3 of ATP-ase. Each reaction tube was kept in the water bath for three hours. At specified intervals 1.0 cm^3 samples were withdrawn and immediately added to Klett tubes already containing 0.1 cm^3 of 50% trichloracetic acid (w/v). This reagent, by poisoning the enzyme stopped the hydrolytic reaction at once. In terms of ATP solution and thromboplastin suspension or their saline solution equivalents, each 1.0 cm^3 sample represented 0.2 cm^3 of 0.0052 M ATP (when present) + 0.2 cm^3 of 30% thromboplastin suspension. Appropriate blanks corresponding to each reaction tube were prepared and read on the Klett-Summerson photoelectric colorimeter at the same time that the respective reaction samples were read.

This experiment was repeated in its entirety and very similar results were obtained in both instances. The data reported in terms of Klett units from one experiment are plotted figure 2. The Klett reading, after it has been corrected by subtracting the appropriate blank reading, is directly proportional to the free phosphate present in solution. Therefore, since the purpose here is merely to demonstrate the effect of thromboplastin on the hydrolysis rate, it has been deemed unnecessary to convert the Klett units into milligrams of free phosphorus. From the curves presented in figure 2, it can be seen that phosphorus is not released from the thromboplastin suspension during incubation whether ATP is present or not, nor does the thromboplastin itself possess ATP-ase activity. Of paramount interest is the demonstration of the thromboplastin inhibition of the enzymatic hydrolysis of ATP when catalyzed by ATP-ase. It may be argued that the inhibition of the reaction might be due to an inactivation of the ATP-ase by thromboplastin. In the light of the previous experiments, however, and the nature of the substances involved, it would seem that the inhibition was due to a binding of the ATP by thromboplastin. The binding of muscle ATP by some protein normally present in muscle has already been suggested by Buchtal, Deutsch and Knappeis ('46) and such a protein could very well be muscle thromboplastin.

The fact that even with excess thromboplastin and calcium chloride, normal clotting in the presence of ATP is either inhibited or delayed, points to the possibility that some other component of the clotting system, such as thrombin, may be affected by the ATP. To test this possibility, the effect of incubating duck thrombin with an ATP solution for three hours was studied. Two reaction tubes were set up in the constant temperature water bath at 38°C. One tube contained 2.0 cm³ of duck thrombin plus 2.0 cm³ of 0.01 M ATP solution. At specified intervals an aliquot of 0.2 cm³ was removed and added to 0.1 cm³ of oxalated duck plasma previously incubated in the water bath for 30 seconds. Three such clotting

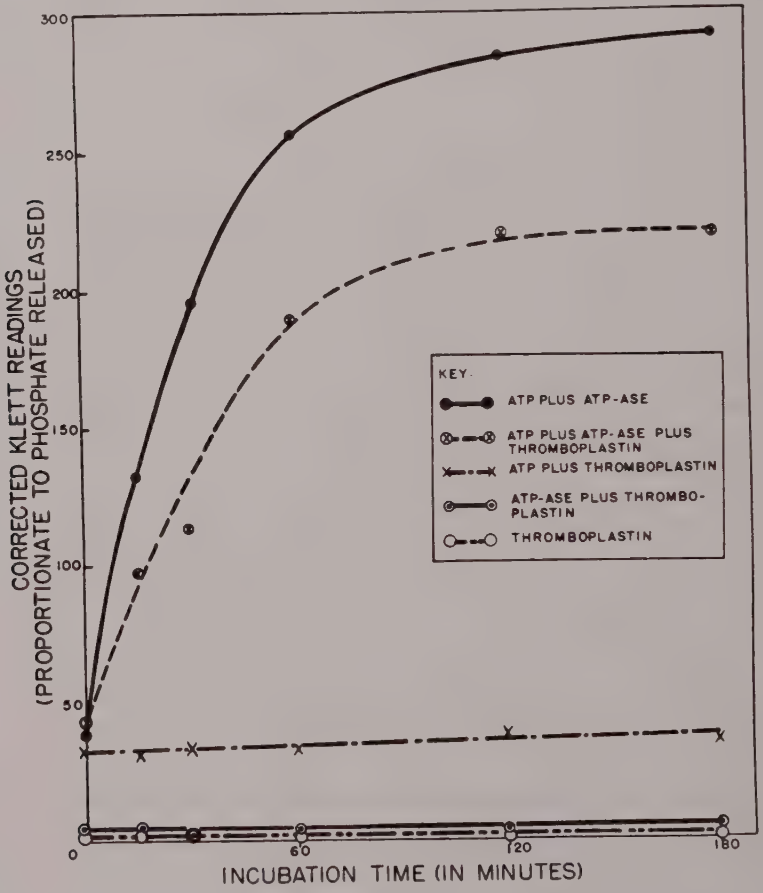

Fig. 2 The effect of thromboplastin on the ATP-ase hydrolysis of ATP.

times were taken for each time interval for each reaction tube. The averages of these determinations are reported in table 5. Comparing these data with those presented in table 3 for the incubation of thromboplastin with ATP, it can be readily seen that there is little progressive binding or inactivation of thrombin as there was in the case of thromboplastin.

To substantiate the conclusion that ATP and thrombin are not mutually inactivating, a hydrolysis experiment involving thrombin, ATP, and ATP-ase was conducted in a manner similar to that previously reported for thromboplastin. Five reaction tubes were set up in the constant temperature bath as before and the rate of phosphate release was determined colorimetrically. From the data obtained from this experiment, it was obvious that thrombin, unlike thromboplastin, did not inhibit the enzymatic hydrolysis of ATP but actually accelerated it. Taken in conjunction with the previously reported experiment (the incubation of ATP with thrombin) it would seem that the ATP has little effect on the clotting activity of thrombin.

TABLE 5

The effect of incubating thrombin with ATP on the clot promoting ability of thrombin on oxalated duck plasma

INCUBATION TIME	AV. CLOTTING TIME WITH THROMBIN PLUS SALINE SOLUTION	AV. CLOTTING TIME WITH THROMBIN PLUS ATP SOLUTION
min.	*sec.*	*sec.*
0	13.9	22.7
30	16.5	24.5
60	18.3	27.9
120	21.2	29.3
180	23.0	30.4

If the anticoagulant action of ATP does not involve the inactivation of thrombin, then perhaps another component of the clotting system, such as fibrinogen might be involved. Since plasma is a source for Ac-globulin, fibrinogen and prothrombin, incubation of plasma with ATP might inactivate anyone of the three or all of them. Plasma, therefore, containing any or all of these inactivated constituents, would show an inhibition of clotting when treated with a thromboplastin-calcium mixture. On the other hand, if plasma were treated with thrombin, inhibition of clotting should occur only if the fibrinogen were affected.

To determine whether ATP inactivated fibrinogen or not, two reaction tubes were incubated for three hours in the constant temperature bath at 38°C. One tube contained 2.0 cm^3 of oxalated duck plasma plus 2.0 cm^3 of saline solution. The other tube contained 2.0 cm^3 of oxalated plasma plus 2.0 cm^3 of 0.01 M ATP solution. At specified intervals 0.2 cm^3 of the reaction mixture was withdrawn and added to 0.1 cm^3 of thrombin previously incubated for 30 seconds.

TABLE 6

The effect on the clotting ability of oxalated duck plasma after incubation with ATP, using thrombin as a clotting agent

INCUBATION TIME	AV. CLOTTING TIME OF PLASMA PLUS SALINE SOLUTION	AV. CLOTTING TIME OF PLASMA PLUS ATP SOLUTION
min.	*sec.*	*sec.*
0	17.8	26.7
30	19.8	32.7
60	22.1	38.1
120	29.8	42.9
180	63.7	63.4

Three such determinations were made for each time interval for each reaction mixture. The results reported in table 6 indicate that ATP had a slight effect on fibrinogen. These results on fibrinogen are in conformity with those of Wunderly ('48) who reported that ATP inhibited slightly the conversion of fibrinogen to fibrin.

Now that thrombin and fibrinogen have been shown to be comparatively unaffected by ATP, the two remaining clotting constituents of plasma (prothrombin and Ac-globulin) must be examined for sensitivity to ATP. In a system consisting of oxalated plasma plus thromboplastin-calcium chloride mixture ± ATP, it would be difficult to differentiate between an effect on prothrombin and Ac-globulin. Murphy and Seegers ('48) report that avian plasma (i.e. chicken plasma) is very low in Ac-globulin and consequently is very sensitive to inactivation, particularly by oxalate and/or storage. Prothrombin, however, is quite stable during storage. Assuming that duck plasma (like chicken plasma) is low in Ac-globulin, an experiment was designed to determine whether ATP affects the Ac-globulin or not. Clotting times were taken in the usual way on samples of fresh oxalated plasma either in the presence of ATP or its absence. The remainder of the plasma was stored for a week in a refrigerator. At the end of that period, samples were again tested as above for clotting activity both in the presence of ATP and its absence. Since avian Ac-globulin is presumed to disappear during storage, stored plasma, whether treated ATP or not, would show the same clotting activity provided that ATP affected only the Ac-globulin. If, however, there were to be a much greater inhibition of clotting in the stored plasma plus ATP than in the stored plasma plus saline, then it could be said that the prothrombin, rather than the Ac-globulin, was affected by the ATP. Experimental evidence demonstrated that the ATP did actually affect the clotting activity of the stored plasma in a manner indicating prothrombin inactivation.

Since prothrombin was inactivated and not the Ac-globulin, it is of interest to determine the rate and degree of its inactivation. To make such determinations, the effect on clotting time was measured on plasma-saline mixture and on a plasma-ATP mixture during a three-hour incubation period.

Two reaction tubes were set up in the constant temperature water bath at 38°C. One contained 2.0 cm^3 of oxalated duck plasma plus 2.0 cm^3 of saline solution. The other contained 2.0 cm^3 of oxalated duck plasma plus 2.0 cm^3 of 0.01 M ATP. At specified intervals, a 0.2 cm^3 sample was withdrawn and placed in a clotting tube. To this was added 0.2 cm^3 of thromboplastin-calcium chloride (0.025 M) mixture, and the clotting time was taken as usual. In most cases three determinations were made for each time interval for each reaction mixture. At those intervals, however, where clotting times were greatly prolonged only two determinations could be made.

The averaged data are presented in figure 3 where it can readily be seen that the inactivation curve rises at a rapid rate. The steepest part of the slope lies between 30 and 60

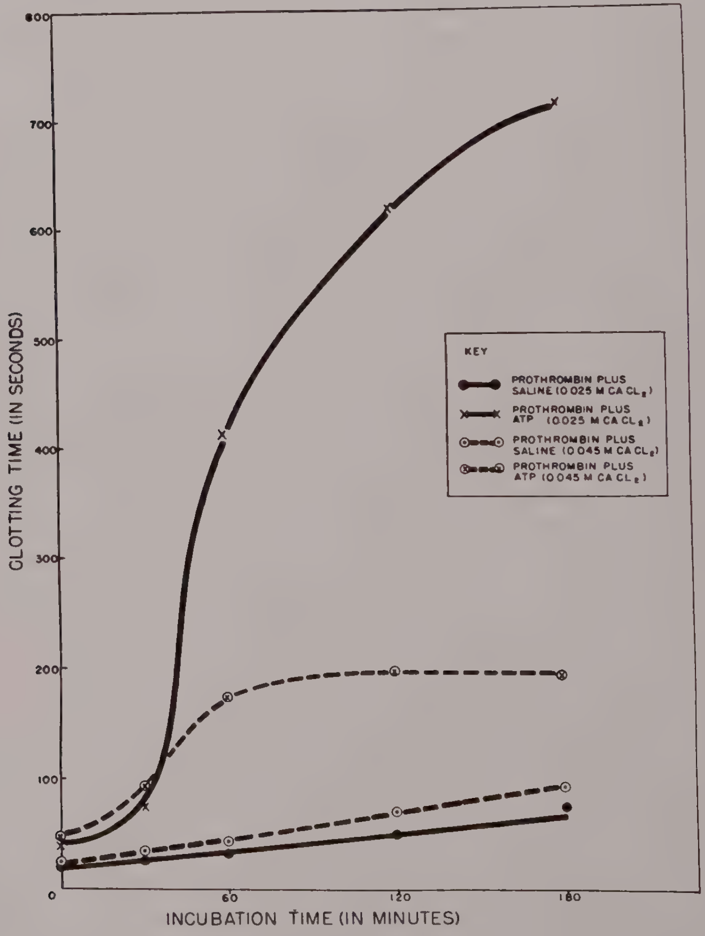

Fig. 3 The effect of incubating oxalated duck plasma with ATP on the clotting time of the plasma (using 0.025 M and 0.045 M $CaCl_2$).

minutes, and at some time shortly after three hours the maximum of the curve is reached.

The results reported above may be interpreted as being due solely to a gradual removal of plasma calcium by the ATP. Using excess calcium chloride (0.045 M) the experiment was repeated, and the results are also plotted in figure 3. The curves presented here show that the additional calcium here (just as in the earlier experiments reported) does suppress the inhibitory action of the ATP, but again the normal clotting time is not restored.

Excluding, for the time being, the question of the action of ATP on calcium, it would seem from the above results that the anticoagulant action of ATP is at least two-fold. Not only does the ATP appear to be able to bind the thromboplastic lipoprotein, thereby inactivating it, but the ATP is apparently also capable of inactivating the prothrombin.

Recently Meister ('48) reported the presence of an ATP-ase, myokinase, and an alkaline phosphomonesterase in human serum. Furthermore, Kay ('28) and Sjöberg ('40) have reported the presence of a pyrophosphatase (= apyrase) in human, horse, dog and beef blood. The latter investigator showed that the enzyme is present in greater abundance in the red blood cell than in the plasma. Nevertheless, there is a measurable amount in the plasma, too. It is therefore conceivable that the prothrombin is not bound to the ATP as the thromboplastin is, but that it is inactivated by some breakdown product formed by the enzymatic degradation of ATP during the three-hour incubation period. Meister points out that there are two pH optima for his enzymes. At the higher pH optimum (pH 8.9) one mole of ATP yields three of phosphate plus one mole of adenosine. At the lower pH optimum (pH 4.8) one mole of ATP yields either one or two moles of phosphate plus one mole of adenosinediphosphate (ADP) or one mole of adenosinemonophosphate (AMP), depending on how much of the ADP is converted to ATP plus AMP by the myokinase. Furthermore, the enzymes involved are at a minimum activity at pH 6.5 to 7.5. Sjöberg, however, found that the pyrophosphatase was most active at a pH of 7.4. The formation of the various phosphates and pyrophosphate would have effects on clotting varying with the solubility of the calcium salt formed. To determine which enzyme system might be operating during the three-hour incubation period, pHs were taken with a Beckman pH meter of the oxalated duck plasma-saline mixture and the oxalated duck plasma-ATP mixture. The pH curves are shown in figure 4. Considering the pH change of the plasma — ATP mixture, it can be seen that if any phosphate is released, only insignificant amounts of $H_2PO_4^-$ and $PO_4^{\equiv}$ could be formed. However, some $HPO_4^=$ and considerable $P_2O_7^{\equiv}$ might be formed during incubation.

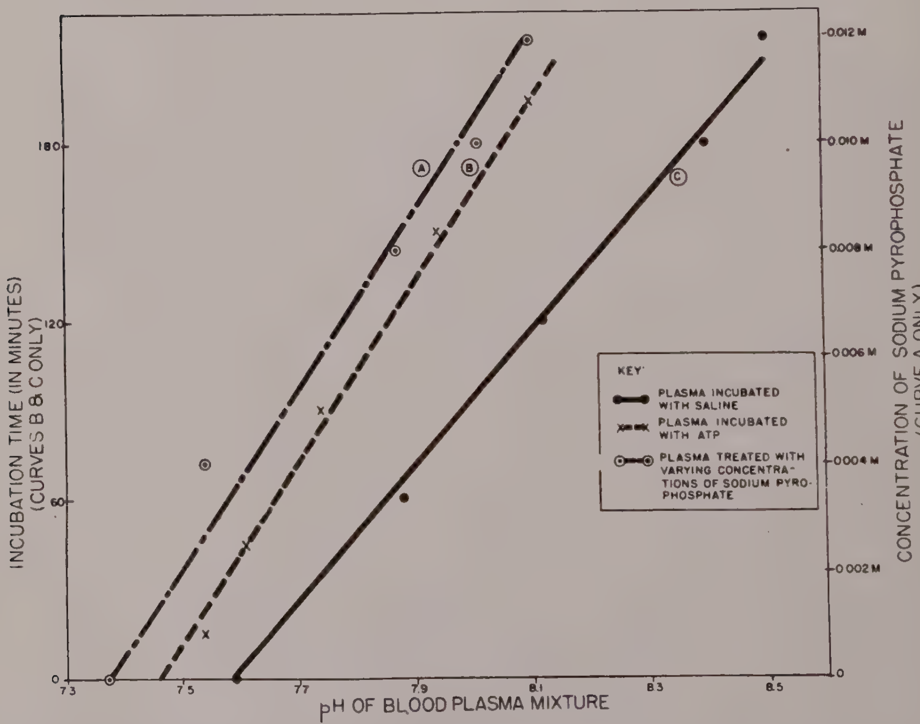

Fig. 4 The effect of the incubation of ATP with plasma on the pH of the plasma mixture compared to the effect of various concentrations of sodium pyrophosphate on the pH of plasma.

To test the effects of these two ions on clotting, a series of dilutions of the sodium salts of each acid was made. Three determinations for each dilution were made as follows:

A clotting tube containing 0.1 cm³ of the dilution being tested was incubated for 30 seconds in a constant temperature water bath at 38°C. Thereafter, 0.2 cm³ of thromboplastin-calcium chloride (0.025 M) was added and the clotting times taken in the usual way. An equivalent volume of saline solution was used for 0.00 M Na_2HPO_4 and 0.00 M $Na_4P_2O_7$.

TABLE 7

The effect of various concentrations of dibasic sodium phosphate (Na_2HPO_4) and sodium pyrophosphate ($Na_4P_2O_7$) on the clotting of oxalated duck plasma

SALT CONC.	AV. CLOTTING TIME WITH Na_2HPO_4	AV. CLOTTING TIME WITH $Na_4P_2O_7$
	sec.	*sec.*
0.000 M	17.6	24.6
0.004	18.2	39.0
0.008	19.1	71.0
0.010	. . .	157.8
0.012	20.2	No clot within 30 min.
0.016	20.6	. . .
0.020	20.1	. . .
0.024	20.3	. . .
0.028	20.0	. . .
0.032	19.6	. . .

From the data reported in table 7 it can be seen that the Na_2HPO_4 had no effect on the clotting time while $Na_4P_2O_7$ had a decided inhibitory effect. Futhermore, if the pH changes involved in the addition of increasing amounts of $Na_4P_2O_7$ to plasma are followed on the pH meter a curve (fig. 4) very similar to that found when incubating plasma with ATP is developed. Over this pH range, $P_2O_7^{\equiv}$ forms an insoluble calcium compound. In fact, Astrup ('50) reports that pyrophosphate is used in Europe as a decalcifying anticoagulant in place of oxalate.

Thus the fact that pyrophosphate is probably liberated on hydrolysis of ATP by the plasma apyrase can readily account for the decalcifying action of ATP. The reduction of this inhibition of clotting by the addition of excess calcium chloride is compatible with these findings. However, indications are that some other substance is also contributing to the anticoagulant action of ATP, since the normal clotting time is never restored even with excess calcium and thromboplastin.

It is possible then, that the other end product formed by ATP hydrolysis i.e. adenylic acid (AMP) might have anticoagulant activity also. This activity would not be that of calcium removal for according to Fiske and SubbaRow ('29) calcium adenylate is soluble. To test the effect of AMP, concentrations of this compound ranging from 0.00 M (where an equivalent volume of saline solution was used) to 0.012 M were used in a clotting system. The experiment was conducted as follows:

TABLE 8

The effect of AMP on the clot promoting activity of thromboplastin and thrombin on oxalated duck plasma

CONC. OF AMP	AV. CLOTTING TIME OF PLASMA PLUS THROMBOPLASTIN-CALCIUM CHLORIDE	AV. CLOTTING TIME OF PLASMA PLUS THROMBIN
	sec.	*sec.*
0.000 M	20.7	11.2
0.002	22.2	10.9
0.004	22.2	11.4
0.006	23.9	10.8
0.008	25.1	11.1
0.010	27.3	10.8
0.012	27.5	11.5

A clotting tube containing 0.1 cm³ of oxalated duck plasma plus 0.1 cm³ of the dilution of AMP to be tested was incubated for 30 seconds in the constant temperature water bath at 38°C. To this was added 0.2 cm³ of thromboplastin-calcium chloride (0.025 M) and the clotting time was taken in the usual way.

Three determinations for each concentration were made and the averages are reported in table 8. It can be seen that the AMP does inhibit clotting but not to the extent that the pyrophosphate does. This inhibition by AMP is in agreement with the work of Polcak ('36) who reported inhibition of clotting when this substance was injected subcutaneously.

Since, in a previous experiment (see table 6), it was shown that ATP inhibited the conversion of fibrinogen to fibrin, it was of interest to see if the effect could be attributed to the AMP released by the pyrophosphatase hydrolysis of ATP. The experiment was carried out as follows:

A clotting tube containing 0.1 cm³ of oxalated duck plasma plus 0.1 cm³ of the AMP concentration used (ranging from 0.00 M to 0.012 M) was incubated for 30 seconds. Thereafter, 0.1 cm³ of thrombin solution was added and the clotting time was taken in the usual way.

Three determinations for each concentration were made and the averages are reported in table 8 also. These data show that the AMP had no effect on the conversion of fibrinogen to fibrin.

If the data of the various experiments reported here are examined, it can be seen that the clotting times for the plasma plus saline mixtures in the presence of 100% thromboplastin suspension varies. This apparent inconsistency may be due to the fact that blood was used from 4 different ducks; or that the dry thromboplastin had aged for several months during the course of this investigation; or that various components of the clotting system were added under varying conditions. This inconsistency is of little importance since all of the results reported are relative and/or the individual experiment was done using a parallel control.

The range of the majority of the values for plasma clotting times fall within ± 5% of the average clotting times reported in tables 1 to 8. A few of the more prolonged clotting times in the presence of ATP have a somewhat greater range. This range, in some cases, approaches approximately ± 12.5% of the average reported.

DISCUSSION

The literature contains many conflicting reports on the effect of ATP on various biological systems. This chemical substance has been applied to such diverse phenomena as muscular contraction (Szent-Györgyi, '51; and Buchtal, Deutsch and Knappeis, '46), mitosis in regenerating liver tissue of rats (Marshak and Walker, '45), cleavage in abnormal sea urchin eggs (Kriszat and Runnström, '51), protoplasmic viscosity in amoeba (Kriszat, '49) and of sea urchin eggs (Runnström and Kriszat, '50). In the cases of muscular contraction, protoplasmic viscosity, and cleavage, ATP apparently promotes protoplasmic gelation. On the other hand, in mitosis and plasma clotting involving similar gelation systems, ATP appears to be inhibitory.

In working with ATP, cognizance should be taken of two important conditions. The first, is that ATP may undoubtedly be contaminated with Ca^{++} or Ba^{++} by virtue of its method of manufacture. These two ions have long been known to promote gelation. The second is the rapidity with which ATP disappears in various tissue preparations (Bollman and Flock, '44; and Humphrey and Humphrey, '50). Several enzymes may be involved in such hydrolytic processes, but the optimum pH of most does not lie in the physiological range. That of the apyrases, found in various tissues by Kay ('28), Barth and Jaeger ('48), and by Krishnan ('49) lies between pH 6.5 and pH 7.6.

Thus, in all probability, adenylic acid and pyrophosphate are released as ATP disappears. Furthermore, Bailey ('49) has shown that in storage ATP is capable of breaking down into AMP and pyrophosphate or phosphate. Regardless of the manner in which ATP is split, the products thus released are very potent physiologically. It is, therefore, possible for investigators to attribute to ATP an effect brought about by either pyrophosphate or AMP.

It has been reported that AMP inhibits cleavage (Druckery, '38), that it relaxes smooth muscle (Robertson and Shaw, '41), causes slowdown of heart rate (Drury and Szent-Györgyi, '29), and inhibits blood coagulation (Polcak, '36). In all of these cases AMP consistently acts as an inhibitor of gelation. Pyrophosphate, on the other hand, is inconsistent in that it inhibits in some instances and in others, it aids. For example, pyrophosphate is used as an anticoagulant for blood and yet it has been reported by Buchtal et al. ('46) to cause contraction of muscle fibres. That pyrophosphate is a powerful physiological agent is further attested by the fact that this chemical when injected is capable of producing many symptoms of the shock syndrome (Mylon et al., '43; and Bielchowsky and Green, '44).

Thus when an effect is obtained with ATP, the possibility that Ca^{++} or Ba^{++} may be present, that tissue enzymes are hydrolyzing the ATP, or that this compound may be partially hydrolyzed to start with must not be overlooked.

In this investigation, the most potent effect that has been obtained with ATP (i.e. inhibition of clotting when ATP is incubated with plasma) is probably attributable to the release of pyrophosphate by a plasma enzyme. Since this effect is not completely nullified by excess calcium, it is thought that the pyrophosphate probably inactivates prothrombin as well as decalcifies the plasma. As has been shown, AMP also has a slight inhibitory effect, but its effect is negligible in comparison to that of pyrophosphate.

Not only has there been shown here an effect due to the breakdown products of ATP, but another effect involving the intact ATP molecule has also been demonstrated. Illustrative of the latter is the binding of ATP by thromboplastin with its subsequent inhibition of coagulation. That ATP, as a whole, is involved here is proven by the fact that there is no phosphate released when ATP and thromboplastin are incubated together.

It can be seen then that ATP may exert physiological effects by virtue both of its own properties and the properties of its breakdown products.

CONCLUSIONS

1. Adenosinetriphosphate (ATP) inhibits blood plasma clotting in both oxalated duck blood and heparinized duck blood.
2. The inhibition is in part due to a decalcification of plasma.
3. Other components of the clotting reaction are also affected.
4. ATP forms a bond with thromboplastin, partially inactivating it.
5. ATP has the ability to inactivate prothrombin when incubated with it. This inactivation is probably due to the release of pyrophosphate which probably acts as a decalcifying agent.
6. ATP has little effect on thrombin and Ac-globulin.
7. ATP or released pyrophosphate may have some role in the slight inhibition of the conversion of fibrinogen to fibrin.
8. Adenosinemonophosphate (AMP) also acts as an inhibitor of plasma clotting but to a lesser extent than ATP.

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The writer wishes to express his appreciation to Dr. L. V. Heilbrunn for the guidance offered during this investigation and for his constructive criticism and suggestions in the preparation of this manuscript.

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MECHANICS OF IODOACETATE RIGOR OF MUSCLE[1]

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FOUR FIGURES

A significant finding for the elucidation of the relation between mechanics and chemistry of muscular contraction was Lundsgaard's discovery ('30a,b; '34) that iodoacetic acid (IAA)- poisoned muscles responding in an activity series, at first produce quite normal contractions, but develop fatigue more rapidly than normally and then become moribund in a process involving loss of excitability and a progressively increasing terminal rigor. The proof that this series of responses occurs in the complete absence of lactic acid formation shattered the foundations of the "lactic acid theory" of contraction so much in vogue during the late 1920's (Hill, '32). But more significant to the present state of muscle research were the demonstrations that in consequence of IAA-inhibition of glycolysis, first creatinphosphate (CP) and then adenosinetriphosphate (ATP) break down irreversibly, with especially the latter occurring in rough parallelism with the growth of rigor. For from these observations and others there developed the conclusions that CP decomposition follows that of ATP and is in fact concerned with resynthesis of the latter, and that thus the energy of CP is used in purely chemical recovery processes, while that of ATP serves to directly energize the contractile material for mechanical work.

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[1] These studies were aided by a contract between the Office of Naval Research and New York University, NR113-300. A preliminary report of them has been given: Abstr. XIX Internat. Physiol. Congress: 727, 1953.

[2] This paper is based on the doctorate thesis of Dr. Schneyer whose present address is University of Alabama School of Dentistry, Birmingham 5, Alabama.

A particularly noteworthy attempt to put this role of ATP into a specific form is the hypothesis proposed by Kalckar ('41, '42). Evidently influenced by the supposition of Needham ('37) and of Lundsgaard ('38) that ATP energy may be used for the restoration of a contracted muscle to its resting state, and impressed by the conditions that a muscle in IAA rigor is exhausted due to the loss of practically its entire store of ATP, and accepting the commonly held view that it is unable to relax, Kalckar ('41) hypothesized that phosphate bond energy of ATP "most likely is used not for contraction but for relaxation of the contracted muscle, and the relaxation is probably the charged state of the contractile system." This interpretation of IAA rigor thus proposes a mechanochemical scheme of the type called "recovery energization", and is opposed to "activity energization" (in which it is believed that energy is delivered to the contractile system as it passes into its active state) (Sandow, '47); and it has thus played a role in the general controversy concerned with the means by which chemical potential energy is transferred to the contractile material for conversion into mechanical work.

But of immediate concern here is that despite interest in the mechanochemical effects of IAA poisoning, and despite also the great amount of work that has been done on the metabolic disturbances due to this inhibitor, relatively little study has been made of the purely mechanical features of muscle in IAA rigor (see, e.g., Godeaux, '50). And it is noteworthy that when observations of certain of these features have been recorded, as, e.g., the fact that IAA rigor is mechanically not irreversible (Lippay and Patzl, '35a, b; Aubert and Rombaut, '53), their significance seems not to have been fully realized. Hence the purpose of the present research is to increase our knowledge in this field in the hope that a more extensive investigation and analysis of the mechanical features of IAA rigor will serve to explain its mechanochemical basis, and thus possibly also of normal contraction itself.

GENERAL METHODS

All of our experiments have been done on the sartorius muscle of the frog (Rana pipiens). After excision each muscle was equilibrated in oxygenated, phosphate-buffered (pH 7.2) Ringer's solution for one hour, and then subjected to some particular treatment involving IAA (or, as in certain experiments, iodoacetamide). The studies were made at room temperature of about 20 to 25° C. Both isotonic and isometric recording were used. The isotonic lever was of the relatively inertialess type and this provided even twitch and tetanus records, which were made on a smoked drum, negligibly free of overshoot. In some of the experiments the isometric lever was of the simple mechanical type having a rather large deflection/tension coefficient to permit ordinary kymographic recording. In other tests, it was of the optical type which allowed not more than 1% shortening of the muscle at tetanus peak; but the optical lever provided sufficient magnification so that the corresponding deflection was on the average about 60 mm. Such deflections in general, i.e., for quick contractions as well as for slowing varying rigor, were not photographed but were recorded by directly noting the position of the light beam focused on a scale. Even for the very quick changes produced by twitches, this method proved adequate since peak deflection which was all that was measured could be read to within about 0.2 mm. The muscles were either mounted in a moist chamber and kept throughly wet by periodically dropping the appropriate solution on them, or they were fully immersed in their test solution in a massive stimulation chamber. In the former case stimulation was effected by condensor discharges of 0.2 msec. time constant, by way of ordinary wire electrodes, while in the latter, square wave massive shocks of 0.1 msec. duration were used. In many of the studies described in the results reference is made to the normal twitch and tetanus tension or shortening. Routinely this was determined on the muscle after its initial equilibration in Ringer's solution, before it was immersed in the test IAA solution.

RESULTS

Before describing our findings in detail, it must be noted that in general here and in all previous work on IAA rigor the results for a given experimental procedure may be quite variable. For that matter, so also are determinations of corresponding chemical changes, as may be seen by reference to various papers of Lundsgaard ('30, '34). Hence, in general, we have not attempted any statistically significant quantification of our data, but instead emphasize only the broad, qualitative differences of interest.

Spontaneous rigor

In the usual procedure for reproducing IAA effects a muscle, after preliminary exposure to the inhibitor, is subjected to some sort of activity series and the rigor then develops in consequence of the imposition of this activity on the already poisoned muscle. We shall refer to this type as "activity rigor." But a muscle will go into rigor when immersed in an IAA solution even though it is not activated at all, provided sufficient time is allowed for action of the inhibitor; and rigor will also appear in a muscle that is first completely fatigued while normal and then, at rest, submitted to the action of IAA. We shall designate by the term "spontaneous rigor" such cases in which the characteristic effects appear when IAA is applied to only the resting muscle whether previously fatigued or not.

Spontaneous rigor in the absence of any activity has been studied by Lippay and Patzl ('35a and b) under both isotonic and isometric conditions. But the limited nature of their research, e.g., the use of only one concentration of IAA (1/20,000, i.e., 0.25 mM), and the presence of certain ambiguities regarding the initial tension in the isometric experiments indicated the need for further studies on this kind of rigor. Furthermore, our own experiments on spontaneous rigor development in normally fatigued muscle are of a type not heretofore reported.

(a) Unloaded rested muscles. In these tests each muscle after equilibrating to normal Ringer's for one hour was freely immersed horizontally in unoxygenated Ringer's containing a given concentration of IAA. With care taken to avoid any activity, the muscle length was measured as a function of time. The results graphed in figure 1 show that in general, irrespective of IAA concentration over the range from 1 to 0.02 mM, the rigor shortening begins after a delay period and then develops to a quite fixed maximum at which the muscles have shortened by about 60% of their original length. As the IAA concentration is decreased, the delay period increases, but the rate of rigor shortening is not affected, except at 0.02 mM where it is greatly reduced. But of immediate interest is that irrespective of the concentration of the IAA the final, spontaneously developed rigor shortening is so great. This decrease in length is just equal to the greatest shortening obtainable in a maximal isotonic tetanus of an unloaded or

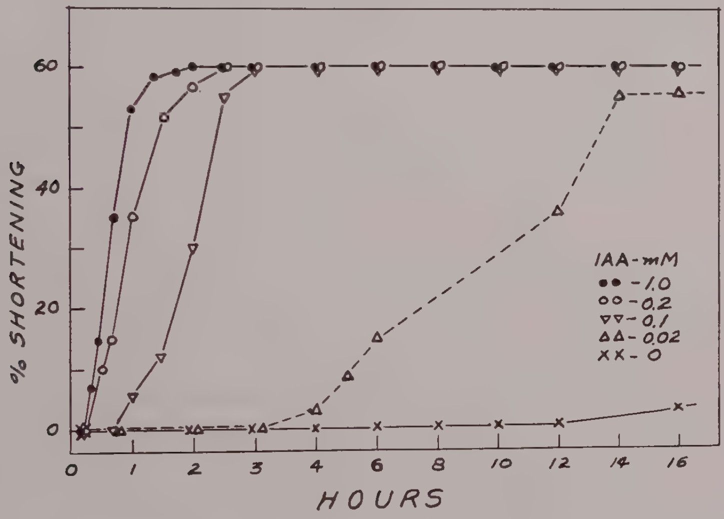

Fig. 1 Spontaneous rigor shortening. Freely immersed (unloaded) muscles. Each curve gives average behavior of 3 muscles in a stated IAA concentration in Ringer's solution. Room temperature; range 20–25°C. Note change of scaling at times greater than 4 hours.

slightly loaded normal muscle. Furthermore, it is noteworthy that this final shortened state is maintained quite indefinitely (e.g., up to at least 24 hours) as long as the muscles are not subjected to any stretching force. I.e., although under the conditions specified above spontaneous shortening occurred, there was no evidence of a subsequent spontaneous lengthening.

Although variation of IAA concentration in the above experiments had no effect on the degree of shortening of the maximally affected muscles, it did introduce differences in both their extensibility and responsiveness. Thus at concentrations above about 0.1 mM, the muscles became relatively inextensible and, especially when they had been in 1 mM IAA, they gave little or no response to electric stimulation. But the muscles subjected to 0.02 mM IAA could be easily stretched out to their normal length and were practically as responsive in a twitch activity series as were muscles that had been soaked for only an hour in this concentration of IAA. These differences were not thoroughly investigated, but the observations that were made indicate that high concentrations of the inhibitor acting for sufficiently long times not only induce spontaneous shortening, as do the lower concentrations, but they also greatly decrease or even completely suppress the capacity of the muscle to respond to electric stimulation.

(b) Loaded rested muscles. In confirmation of work by Lippay and Patzl ('35a), we have found that spontaneous rigor appears also in muscles pulling against a load by being connected to either isotonic or isometric levers. In this type of test, the muscle was in some experiments mounted in a moist chamber and perfused with the IAA solution at a steady rate of 1 drop a second, while in others it was fully immersed in the test solution. Either procedure gave essentially the same general results in that the rigor shortening or tension, after an initial delay period, develops moderately slowly to a maximum and then, under continuing action of the load, gradually disappears, this whole process occurring more slowly, the smaller the IAA concentration. But, as also found by

Lippay and Patzl ('35b) and as recently discussed by Aubert and Rombaut ('53), the rigor output at maximum is several times greater for muscles in the moist chamber than for those immersed in the test solution. This difference is being further investigated in our laboratory and a more detailed presentation of such results will be postponed for later publication.

For detailed analysis here we find of interest our experiments in which the muscles were immersed in the test solution which, in order to hasten the course of the rigor changes, contained IAA at the relatively high concentration of 1 mM. These muscles were connected to the optical isometric lever at an initial tension of 1 gm and after the tests for normal twitch and tetanus tension, the muscles were allowed to rest for about 30 minutes in oxygenated Ringer's in order to recover from this activity before being subjected to the 1 mM IAA test solution.

Some typical results of such experiments are shown in figure 2. Despite the evident variations in the two curves presented, it is clear that after a certain delay rigor develops first in a rather slow phase which lasts about five hours and reaches a tension of about 3% of normal tetanus output. The rigor then rises relatively rapidly during the next 2 hours achieving at peak 16-17% of tetanus strength and following this it declines smoothly until finally all rigor tension disappears. Other muscles similarly treated give essentially similar results.

(c) Normally fatigued muscles. In these studies the muscle is first completely fatigued in a 1 per second series of maximal twitches. Such muscles may develop a slight contracture early in the set of responses, but this is then reversed so that at the time of total fatigue the muscles are as relaxed as they were at the beginning of the activity.

We have performed experiments on such fatigued muscles under both isometric and isotonic conditions, and when exposed to various concentrations of IAA. But the essential effects will be evident if we limit ourselves to the tests in which, except for the initial fatigue, the conditions were identical to those of the preceding section, i.e., optical isometric recording with an initial tension of 1 gm, and IAA at 1 mM. Results of two such experiments are graphed in figure 2, and it is of interest to compare them with the similar data obtained from unfatigued muscles which are also plotted in this figure. It will be noted that both types of muscle develop rigor tension to a common peak value which amounts to about 16-17% of the normal isometric tetanus output, and that the kinetics of rise to this peak occurs in two rather distinct phases as indicated by the change in rate that takes place, though in various forms, when the tension has reached 3 to 5% level of the reference value. During the second phase both kinds of muscle increase their rigor tension at about the same rate. The principal differences between the two types of muscle are then found in the earlier onset of rigor in the

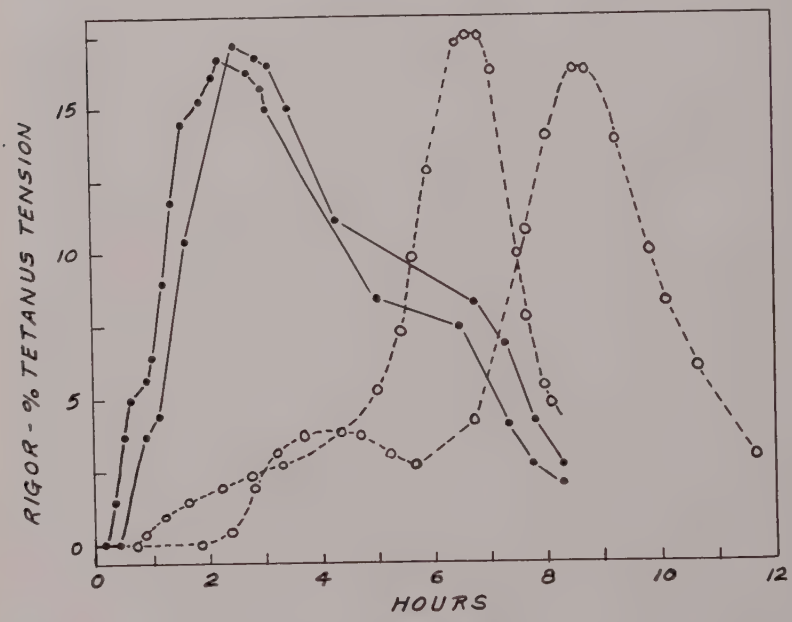

Fig. 2 Spontaneous rigor tension. Typical results of muscles in 1 mM IAA 20°C. Dashed curves: muscles at rest throughout experiment. Solid curves: muscles fully fatigued in a twitch series before exposure to IAA.

muscle that is fatigued before exposure to the IAA, and in the fact that completion of the first phase of rigor development takes place in only about 20 minutes for this muscle, but in about 5 hours for the unfatigued muscle. From these results it would thus appear that the process of development of the bulk of the rigor, i.e., that which appears in the second phase, is essentially the same, both in rate of rise and in peak value attained whether or not the muscle is rested, and that therefore the effect of first fatiguing a muscle before permitting it to react to IAA is to hasten the processes concerned with determining both the onset and the development of the first phase of rise of rigor.

In final comment on these results, it is of interest that in contrast to the failure of the unloaded muscles to relengthen after their rigor shortening, loaded, rigor muscles, whether previously fatigued or not, always relax their tension if isometric or relengthen if isotonic.

Activity rigor

Although as just indicated tension output is quite small in spontaneous rigor, the work of Lippay and Patzl ('35a) proved that it is considerably larger in activity rigor. Their experiments, however, were done with muscle at only one unspecified initial tension; hence, it was considered desirable to reinvestigate this problem under conditions of widely varied initial tension.

Consideration was first given to the question of optimal conditions for production of activity rigor tension. In part, the studies on spontaneous rigor served for this purpose, since, as is evident in figures 1 and 2, some rigor output could appear during the period of soaking in the inhibitor; and tests proved, especially if the IAA concentrations were high, that such spontaneous effects tended to reduce the amount of rigor subsequently generated in conjunction with activity. The procedure found suitable for our needs was to soak a muscle in 0.1 mM or in some experiments 0.2 mM IAA for one hour.

For under these conditions, although some spontaneously developed shortening appeared in freely immersed muscles, the spontaneous tension output was zero in muscles soaked in the IAA solution while connected to the isometric lever under all initial tensions of our tests.

Our procedure was advantageous in certain other respects. Meyerhof and Boyland ('31) studied the diffusion of IAA into the frog sartorius and found at 18° C. that the whole muscle (i.e., both intracellular and extracellular space) was quite fully saturated with the inhibitor after only 30 minutes immersion in the test solution. Furthermore, these workers also demonstrated that after one hour of exposure to 0.25 mM IAA solution glycolysis was practically completely blocked even though respiration was hardly affected. Hence, in consequence of our soaking procedure we may be certain that our muscles were in diffusion equilibrium with the external IAA and that, at least for those cases in which the concentration of the inhibitor was 0.2 mM, there obtained essentially complete inhibition of lactic acid production. It should be noted in regard to the effect on metabolism, that most of our experiments were performed with the IAA at a concentration of 0.1 mM which might not have been as effective as the higher concentration used by Meyerhof and Boyland. However, control tests proved that this concentration of the inhibitor induced just as much activity rigor as did the higher concentration.

The lever system and the method of observation of tension were as described for the spontaneous rigor studies of normally fatigued muscles. In most of the experiments full fatigue was effected in a maximal twitch series, and in these the initial tension was varied; in some others this was accomplished by a long tetanus, but at only one initial tension. In any one run, the normal muscle was set at a given initial tension in the massive stimulating bath and after the usual tests for maximal twitch and tetanus output it was relaxed to slackness while equilibrating to the IAA solution. At the beginning of the activity the initial tension was restored. Following activity and fatigue, rigor was permited to develop while the muscle was still immersed in the IAA medium, and it is the value of rigor at plateau which was recorded for comparison with normal contraction output.

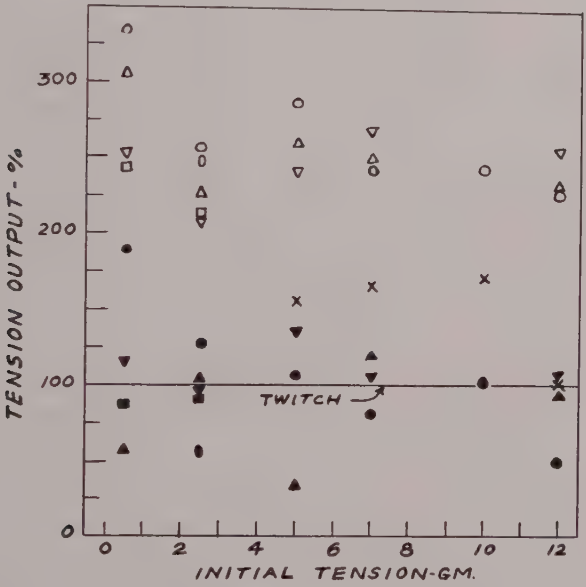

Fig. 3 Maximal activity rigor tension, following a twitch fatigue series, relative to normal twitch and tetanus tension; symbols of the same shape refer to the same muscle. Crosses: rigor tension of muscles for which no tetanus output was determined.

(a) Twitch activity. The results of all such experiments are given in figure 3 and it is evident that despite considerable scatter of the data the tension of maximal activity rigor is, at all initial tensions, only about 1/3 of that of the corresponding tetanus, so that it is about equal to the associated twitch output.[3] Though twitch-activity rigor is still small as com-

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[3] Buchthal ('42) has demonstrated that in single striated muscle fibers, the elasticity modulus, referred to the same tension, is for the state of IAA rigor less than, but for fatigued normal tetanus greater than, that for rest. In a personal communication Dr. Buchthal points out that this finding is consistent with the fact that maximal, muscle output of tension of rigor is less than that of tetanus. However, it should be noted that by use of the method of Gasser and Hill ('24) it has been shown (Sandow and Brust, '46) that after activity the elasticity coefficient of a muscle in rigor is in general greater than it is in the normal muscle.

pared to normal contractile strength, it is evident (by comparing peak rigor of the graphs of figure 2 with the data of figure 3 obtained at comparable initial tension) that it is larger, by a factor of rougly 2X, than the maximal spontaneous rigor. Thus these results are in accord with those of Lippay and Patzl ('35a), but they are more extensive in also showing that the finding of these workers for activity rigor holds irrespective of the initial tension at which the muscle is tested.

In addition to these isometric experiments on twitch-activity rigor, we have made a brief investigation on the effect of after load on shortening in corresponding isotonic studies. In summary, these experiments demonstrate that if the load is quite small—of the order of 0.5 gm—the maximal rigor shortening is equal to that of normal tetanus, as we have shown it is even for the resting, rigor muscle; but as the load is increased, the degree of this shortening becomes a progressively smaller fraction of that obtained in the associated tetanus, so that at an after load of about 12-14 gm no rigor shortening at all occurs. Evidently this inability to shorten much in pulling against the relatively larger loads reflects in another context the condition of a muscle in full rigor that prevents it from producing as much tension as it can in normal tetanus.

In connection with these experiments, it was of interest to determine whether a muscle that had been brought to a state of maximum activity rigor tension could still shorten when its loading was changed from isometric to isotonic. To study this point, a few tests were made as follows. Each muscle, equilibrated to 0.1 mA IAA, was connected to an isotonic lever at an initial load of 2 gm and an after load of 12 or 14 gm and then activated to full fatigue. Such muscles will in due time develop maximum activity rigor, and this will be isometric since the developed tension is too small to lift the relatively large after load. If now the load is reduced to only 2 gm, so that the tension is adequate to lift the load, shortening does occur. But on the average this amounted to only 2 mm which is about 7% of the muscle length and considerably smaller than that produced in activity rigor shortening when the after load is maintained at 2 gm throughout the experiment. Hence, these results show that when muscle is in isometric rigor, reduction of the capacity for tension output also involves an adverse effect on the associated potentiality to shorten.

Finally, it is important to note that the activity rigor of these exepriments is self-reversing. The rate of this relaxation is variable; but, generally, under isometric conditions, the activity rigor may be fully maintained for only a few minutes before relaxation has its onset,[4] and it will continue to disappear at first rather rapidly and then more slowly, with the whole process of reversal occurring at 20° C. in a few hours. Under isotonic conditions, the course of reversal is even more variable both in time of onset, rate of development, and even in respect to the amount of relaxation finally attained (some muscles failing to reach their initial length while others are extended beyond it). Although insufficient study of this isotonic reversal has been made, and further investigation is required, the main point seems to be that the greater the load, the speedier and the more extensive is the relaxation.

(b) Tetanus activity. A number of isometric experiments have been performed, all at an initial tension of 2 gm, in which the poisoned muscle, immersed in its 0.1 mM IAA solution in the massive shock bath, was stimulated to full fatigue by a prolonged maximal tetanus shock (60/sec.). In general, as shown in figure 4, such muscles begin by producing a peak tension, which is of essentially normal strength. Within a period of a few seconds the tension starts to decline and continues to fall progressively for some 10 to 15 sec. until the muscle is about 90 to 95% relaxed, at which time stimulation ends. Rigor then sets in abruptly, climbs rapidly to a maximum which is maintained for a minute or two and then relaxation sets in, developing at first quickly and then more slowly

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[4] It is a pleasure to acknowledge our indebtedness to Mr. George E. Mauriello of this laboratory who first called our attention to the earliness of onset of relaxation of muscle in isometric rigor.

until restoration of the initial tension is attained several hours later. In six such experiments the average maximum rigor tension was 57% of the normal tetanus output (range, 46 to 65%). This may be significantly higher than the corresponding value of the twitch activity rigor experiments, but no attempt was made in the present work to prove this by adequate statistical control. For the present, of greater interest to us is the great rapidity with which the rigor changes run their course, especially noteworthy being the quick onset of relaxation from rigor and the relatively great rate at which it begins to develop.

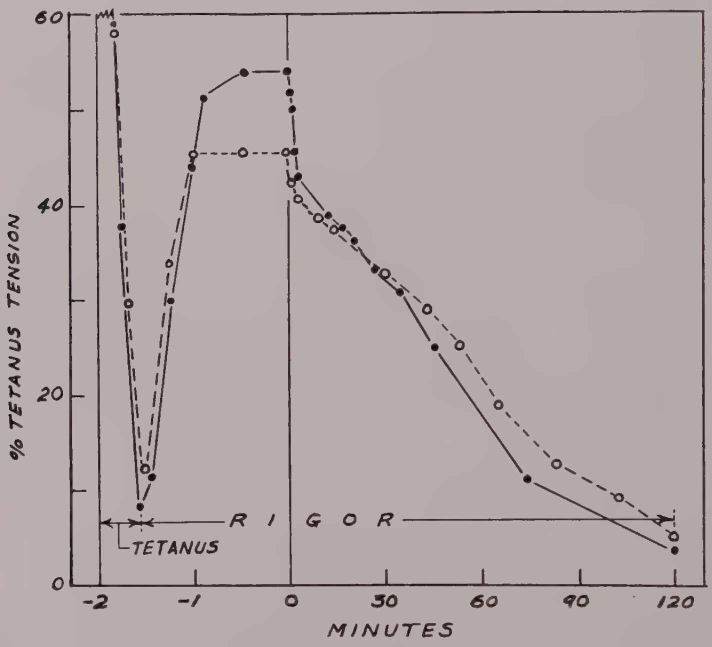

Fig. 4 Course of activity rigor tension following a prolonged tetanus in 2 typical experiments. 0.1 mM IAA. 20°C. Zero of time arbitrarily set at onset of relaxation from rigor. Note difference in time scaling between the negative phase (including tetanus and rigor development) and the positive phase (showing relaxation from rigor).

It is now evident, taking into account all of our work on spontaneous as well as activity rigor—that a muscle, if loaded, does not maintain rigor indefinitely, but always shows relaxation from it, which in certain cases, notably isometric tetanus activity rigor, is quite rapid and complete. These results on reversal of rigor are in accord with and extensions of earlier work (Lippay and Patzl, '35a, b; Aubert and Rombaut, '53). But their significance is made clear by taking into account the new results described in an earlier section of our paper which prove that rigor shortening does not reverse in freely immersed, i.e., unloaded muscle. For this indicates that lengthening of muscle after attainment of rigor is not an active process, but that it involves a passive relaxation mechanism whose expression as a lengthening of the muscle requires the presence of an external stretching force.

DISCUSSION

In previous attempts to explain the nature of IAA rigor [5] two main features are involved: one is concerned with observable mechanical behavior, and the other quite speculatively deals with the relation of the mechanical and associated chemical changes. These two facets of the problem are present in Kalckar's ('41, '42) hypothesis of mechanochemical coupling already outlined in the introduction. In so far as the simple mechanical features are involved, they were explicitly described by Kalckar ('41, p. 73) as follows: "After forty or fifty single contractions a muscle poisoned with iodoacetic acid is exhausted, the contractions decrease rapidly, and simultaneously the relaxation becomes more and more incomplete, i.e., the muscle is completely exhausted in a state of rigor." In view of the belief that rigor develops because relaxation is impaired and that in association with this the muscle's store of ATP is severely depleted, it was logical to hypothesize that the failure of relaxation was due to the absence of a high content of ATP, and that thus in a response the energy of ATP was directly needed to drive relaxation and not contraction (see introduction). At any rate, it is noteworthy that closely connected with the earlier descriptions of the mechanics of IAA rigor are the generally prevalent views that the rigor develops in an exhausted muscle and that it is irreversible. [See, e.g., Wilhelmi ('46, p. 57), Mommaerts ('50, p. 16), and especially Godeaux ('50, p. 907) who states that IAA rigor involves "un déficit croissant et irréversible du relâchement."].

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[5] It should be noted that a confusing feature of the literature concerned with IAA rigor is the designation of it by inappropriate terms, e.g., "contracture" and "tonus" (Godeaux, '50; Ling, '52). We have attempted to clarify our meaning by definition of various terms as follows: *contractile change* — any inherently developed mechanical alteration that results in development of tension or shortening; *contraction* (at least when used for living muscle) — a change such as a twitch or tetanus that is activated in consequence of excitation. As for *rigor* — we are here concerned only with that due to IAA, and our entire paper attempts a definition of it.

Now, in certain features of its behavior, a muscle in IAA rigor is irreversibly changed: the loss of excitability cannot be undone by any means known at present, nor can the metabolic disturbances. It is of interest in this respect that, as originally demonstrated by Lundsgaard ('30b) and confirmed by us in the course of our work, once a resting muscle has been thoroughly exposed to IAA, a subsequent soaking in Ringer (either pure or even containing 0.01 M cysteine) for as long as three hours will not prevent development of typical rigor effects when the muscle is finally activated. Such results, however, are explained by the demonstration (Meyerhof and Boyland, '31) that practically all of the IAA taken up by a muscle soaked in solution of the inhibitor remains irreversibly bound to it when it is returned to pure Ringer.

But, purely mechanically,[6] rigor is spontaneously reversible as demonstrated by many of our results as well as those of others. It might be said that such effects in experiments on spontaneous rigor, which occur after many hours of immersion of a muscle in an IAA solution, result from autolysis or bacterial decomposition of the contractile material.[7] But this is precluded by the fact that in many of our isometric, activity rigor experiments, involving especially tetanic activity (fig. 4), the rigor begins a rapid disappearance in as little as 2 minutes following initiation of its development. Thus, in general, the lessening of tension, or the relengthening, of a muscle that occurs sooner or later from a state of rigor is a property of the contractile material modified only the effects of IAA poisoning. Such relaxation, however is not active since it does not occur unless the muscle is loaded; and under this condition it occurs faster, the greater the load as indicated particularly in the isometric behavior (fig. 4) at least at the onset of relaxation when the tension exerted by the lever system on the muscle is greatest. Hence, relaxation from rigor is passive, as it is during relaxation of a normal contraction (see Hill, '49b).

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[6] It should be emphasized that in this analysis of the mechanical behavior of IAA rigor, we are concerned only with the directly observable changes in length or tension of the muscle. Thus, the use of the term, "relaxation," in describing mechanical reversal of rigor implies only that a muscle previously brought to a state of rigor tension (or shortening) reverts, or tends to revert, to its initial state of lesser tension (or greater length).

[7] That bacterial decomposition is not involved is indicated in preliminary experiments which prove that 1 mM IAA is a quite effective bacteriostatic agent.

The fact that a muscle can relax after rigor development is very important for it suggests that the process that builds up the rigor is itself not due to a mounting failure to relax. Independent proof for this view is found in our results and in those of others that onset of rigor in practically all cases does not require the presence in a muscle of a state of contraction which would be the case if rigor represented an impairment of relaxation. Our results show (figs. 1 and 2) that even in the complete absence of any contraction, spontaneous rigor develops from a completely relaxed state. Moreover, when contraction is evoked in a muscle already fully poisoned with IAA, as in our activity rigor experiments involving prolonged tetanus (fig. 4), the still stimulated though fatiguing muscle first relaxes practically completely before rigor has onset. Hence, we conclude that generation of rigor depends on activating a contractile change and not on suppression of relaxation.

In view of these results, some of which have been long known, it is difficult to see how the misconception arose that IAA rigor develops as an inability to relax. It may be that records of activity rigor developing in a twitch series—such as the one published by Lundsgaard ('30b) — had something to do with this. For in these the rise of the rigor gives an appearance of an increasing failure of relaxation in each twitch. But a close study of many such records reveals that each twitch, except possibly those produced late in rigor development, shows a perfect relaxation to the level of shortening from which it had started, and that the increasing rigor occurs in a series of increments between the twitches. Furthermore, in many experiments of this type performed by us, the variability is such that many IAA muscles culminate a twitch series in a quite perfectly relaxed fatigue, as do our previously discussed tetanized muscle, before any sign of rigor is evident.

From all the foregoing, it is clear that in so far as observable mechanical behavior is concerned, a complete cycle of IAA rigor is in outline like a normal contraction, i.e., in both a contractile change develops and this is followed by a relaxation. In support of this conclusion are the following: (1) our results which show that under proper conditions rigor shortening is as great as in a normal tetanus; (2) Aubert's finding ('53; see also Aubert and Rombaut, '53) that a muscle in rigor has normal-body thermoelastic properties, as it does in contraction as Hill ('53) has shown; (3) the structure of the contractile protein in rigor, as shown by light scattering (von Muralt, '34; von Bayer and von Muralt, '34) and by electrophoresis of the extracted proteins (Dubuisson, '50), is like that of the substance in contraction; and (4) our previously mentioned demonstration that the relaxation from both rigor and contraction are passive. But there are also differences, of a quantitative nature, between the two types of contractile cycles: (1) the course of rigor is obviously very much slower, requiring at best several hours for completion while a contraction runs its course in a time as quick as 0.1 second [yet, rise of rigor to peak may occur in as little as 20 or 30 seconds (see figure 4)]; and (2) the maximal tension of rigor, though about the same as in a twitch, is much less than can be produced in a tetanus (figure 3).

Taken as a whole, all these characteristics suggest that when a muscle goes into IAA rigor, its contractile components are transformed into an active state (Hill, '49a, '53) qualitatively similar to what they possess in normal contraction, but lasting for a much longer time and of very much slower kinetics. In normal contraction, however, the intrinsic strength and the capacity to shorten of the fully activated contractile components are such that, provided the fully active state is sufficiently prolonged, as by a series of closely spaced stimuli, the muscle can produce maximal tetanus tension. But the muscle in rigor, even though it can shorten maximally (at least when lightly loaded) and does maintain its active state for a long time, is not capable of generating tetanus tension. It is not known whether this is due to a lower intrinsic strength of the activated contractile components in rigor than in contraction, or to some other disturbance in the mechanism by which muscle produces tension. Further work is needed on this problem, and clarification of it may be provided by projected, quick-stretch experiments on muscles in rigor.

Turning now to mechanochemical questions, it is evident that, since IAA rigor is subject to automatic relaxation and is developed as an active contractile change, the commonly held view can no longer be sustained that this rigor develops irreversibly because insufficient energy of ATP is available to drive relaxation. Our results strongly indicate, rather, that if ATP reactions are at all involved, the rigor is generated in consequence of the accompanying uncontrolled breakdown of ATP, and that the ensuing relaxation—since it occurs when the muscle's ATP content is very low—does not depend on energy of ATP breakdown but on some other reaction which probably may not even have to release energy to drive the relaxation process.

Further evidence in support of the above is found in a more detailed consideration of the variations in ATP content of muscle in rigor. Lundsgaard's ('30b) determinations show that in activity rigor of frog skeletal muscle at room temperature there is, on the average, at the very onset of rigor about 50% of the normal ATP content, and at maximum of rigor, 27%. This decrease in ATP takes place in a time of the order of 3 min., so that the average rate of decrease is about 10%/min. Thus during the build up of rigor, considerable amounts of ATP disappear at a high rate and the energy so liberated could conceivably be utilized for the mechanical work of this period. However, even when rigor has stopped increasing there is still intact about 30% of the original ATP. But, Hsu ('50) found that his experimental muscles (at 10°C.) lost an average additional 22% of their ATP while maintaining maximal rigor for about an hour. And, although he inferred from this that ATP breakdown results from rather than causes rigor, his finding is otherwise significant to us.

The rate of disappearance of ATP during this period is of the order of 0.3%/min., or correcting for our temperature of about 25°, say 1%/min., i.e., much less than holds for the duration of rigor development. And it is reasonable to assume that this accounts for the absence of any mechanical change. Indeed, these results in general are quite like those Bendall ('51) has found for rabbit muscle, in which rigor mortis has its onset when the ATP level has fallen to about 80% of the normal, rises to a maximum as the ATP rapidly falls to 20-25%, and then shows no further change as the ATP continues slowly to breakdown.

Other indications concerning the mechanochemical relations of IAA rigor are obtained by reference to work on various protein models of muscle. In studies of glycerinated rabbit psoas fibers Bendall ('53) and Bozler and Prince ('53) have both shown that their preparations contract in the presence of ATP when the ATPase activity is high, but if by experimental treatment it is made sufficiently low, the contracted state may be either maintained or give way to relaxation. Thus ATP breakdown in these fibers does not of itself guarantee shortening. And Bendall emphasizes that the relaxation he observes is due to the action of the special relaxing (i.e., the M-B) factor that he and Marsh ('52) have studied. This factor indeed requires the presence of ATP and yet it also reduces ATPase activity as necessary conditions for the performance of its relaxation function. Hence, if these results are applicable to relatively more intact muscle maintaining rigor, or relaxing from it, they indicate that any low residual breakdown of ATP is not involved essentially in causing either contraction or relaxation, and that a low concentration of ATP, as such, may even be capable of causing relaxation. The force of this analysis is then to shift the role of ATP breakdown entirely away from the process of relaxation, and to place it directly in the reaction responsible for causing contractile change, as we do in accounting for the role of ATP in rigor development.

Many other results of the behavior of muscle extractive models are in harmony with our view. Thus all of the work of both the Weber school (Weber and Portzehl, '52) and of Hayashi and Rosenblueth ('52) agree that ATP breakdown causes contractile activity of their preparations and that relaxation is determined by other reactions. Especially interesting is the Weber finding that relaxation occurs by a plasticising action of the pyrophosphate radical (either inorganic or as part of ATP) on the contractile protein. The actual relation between this function and the similar one of the M-B factor is at present unknown, and nothing is known to indicate whether or not the M-B factor is involved in relaxation from rigor. But this relaxation may be caused by the action of ATP-pyrophosphate; for when it is in process, ATP is still present and the rate of its breakdown is so low that in effect a definite though small concentration of the intact nucleotide persists in the IAA muscle and this could provide ATP-pyrophosphate to cause relaxation.[8]

The above discussion showing either actual or inferred functional similarities between the contractile material of a muscle in rigor and as organized after various extraction procedures, suggests that the muscle in rigor may be considered to be a new "model" of the living muscle. As such, its behavior suggests that, if energy of ATP breakdown is involved in normal contraction, it is used directly to drive contraction, and is not utilized to "recharge" the muscle during relaxation. And this view of energy relations holds independently of any comparison of rigor muscle with extracted contractile protein, when account is taken that rigor develops not as a mounting irreversible relaxation, but as an active contractile change.

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[8] This inference is corroborated by the finding (Sandow and Mauriello, '54) that sodium pyrophosphate greatly accelerates the relaxation of muscle from rigor. And this result offers additional proof that relaxation of rigor does not require expenditure of energy derived from the usual type of exergonic metabolic reaction involving decomposition of an organic compound such as ATP.

Brief comment will now be made on differences in the kinetics of rigor development found in our results. The speed with which rigor develops must, in general, depend on: (1) the time for diffusion of IAA to the susceptible loci of the triose dehydrogenase that is inhibited by the poison, (2) the time-reaction involved in suppressing glycolysis (Lundsgaard, '30b), and (3) the speed with which the effects of glycolytic inhibition are relayed by way of CP reactions to the irreversible breakdown of ATP, which apparently is then responsible for causing rigor production. In addition, our particular experimental procedures introduce other factors, such as: IAA concentration, isotonic versus isometric loading, and temporal ordering of activity and exposure to the inhibitor. Reference to our figures 1, 2, and 4 clearly shows that variation of our experimental conditions produces great differences in kinetics of rigor development; and, in connection with the general problem of the mechanochemistry of rigor it would be of interest in such cases to obtain definite data as to whether there is a positive correlation between the rates of ATP breakdown and rigor development. In the absence, at present, of any such detailed data, only some general points will be discussed.

As might be expected, rigor, as in our unloaded, spontaneous rigor experiments (fig. 1), develops more rapidly, the greater the IAA concentration. Yet it is noteworthy that although the rate is very much greater in 0.1

mM IAA than it is in 0.02 mM, it does not further increase very much at concentrations greater than 0.1 mM. These results probably reflect the fact that the experiments using the three higher concentrations involved the IAA at a concentration near to, or greater than, that (0.25 mM) at which Meyerhof and Boyland ('31) proved glycolysis to be completely inhibited. Thus at 0.02 mM IAA rigor developed very slowly since at no time was glycolysis completely suppressed.

Activity, in general, greatly speeds rigor production. This is so even for the muscle that is activated while normal and then poisoned (fig. 2), and in which there must be an unavoidable lag in generation of IAA mechanical effects due to the time taken for three general processes (diffusion, etc.), mentioned above, to run their course in the already fatigued muscle. However, in the normal muscle Lohmann ('28) proved that activation to fatigue results in a 50 to 60% loss of its ATP, so that in this respect the muscle is already at rigor threshold—and then when IAA is added, and ATP soon further breaks down, rigor becomes manifest relatively rapidly. The most rapid rise of rigor is found in activity rigor, and especially when the activity is a long tetanus. In such cases the great speed must be at least in part accounted for by the fact that when activity occurs glycolysis is already completely inhibited and there remains then only the time needed for the activity to cause the great, irreversible breakdown of ATP that engenders rigor.

Finally, some effects of load are of interest. Comparing results of spontaneous rigor due to 1 mM IAA, it is noted that unloaded shortening (fig. 1) is completed in about an hour, but peak isometric tension (fig. 2) is attained only after somewhat more than 2 hours. This indicates that, in rigor, tension develops more slowly than shortening. But this evidently holds only for spontaneous rigor, since as shown in figure 4, activity rigor tension can develop very rapidly. There is at present no explanation for these differences.

SUMMARY

1. All experiments have been done on frog sartorii at room temperature. Unloaded muscle, freely immersed in Ringer's solution containing iodoacetate in concentrations from 1 to 0.02 mM, develop spontaneous (unactivated) rigor causing ultimately a 60% decrease in muscle length, which is equal to that maximally obtainable in normal tetanus; the final shortening is maintained essentially indefinitely unless an external stretching force is applied. As the IAA is decreased to 0.1 mM, the delay of rigor onset is increased up to 40 min., but the ensuing rate of rigor shortening is practically unaffected; in 0.02 mM IAA the delay is increased to 3 hr. and the rigor rate is greatly decreased.

2. In 1 mM IAA, isometrically loaded muscles develop spontaneous rigor tension more slowly than unloaded shortening; after reaching a peak of about 1/6 of normal maximal tetanus tension, they relax slowly to their state of initial tension. When such isometric but normal muscles are first fully fatigued and then placed in 1 mM IAA, they develop spontaneous rigor tension to the same 1/6 level and then relax, in an overall time course, faster especially in onset of the rigor development phase than when unactivated.

3. Activity rigor (resulting from concomitant action of contraction activity and IAA poisoning) produces, under isometric conditions involving initial tension from a 0.2 to 12 gm and a twitch fatiguing series, an average maximal tension of about 1/3 normal tetanus value. This isometric rigor also relaxes after attainment of peak. And when activity is a prolonged tetanus, the fatiguing muscle first relaxes to practically its initial tension, and then after very quickly developing maximal rigor, it quite rapidly begins and more slowly continues to relax to its initial tension.

4. The results prove that IAA rigor is incorrectly described as an irreversibly mounting failure to relax, but that it involves a cycle of an active contractile process followed by a relaxation, and thus it is comparable mechanically to an

ordinary contraction. The state of rigor is discussed in relation to active state mechanisms of normal contraction.

5. Analysis of the results in relation to known chemical changes associated with the IAA rigor cycle and to properties of contractile models composed of muscle extractives, leads to the inference that the mechanical energy expended in rigor development is obtained from breakdown of adenosinetriphosphate and that relaxation from rigor is not dependent on this reaction.

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